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Rapid Induction of lipids and harvesting of microalgae for biofuel and high value products.

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Abstract

Microalgae have been considered as a potential feedstock for sustainable production of biofuel, animal and aquaculture feed, antioxidants and bioactive compounds. These organisms are the primary producers of organic matter in aquatic environments due to their photosynthetic activities; they possess several advantages when compared to terrestrial plants. However, commercial scale microalgae application is rarely economical due to slow-growing high-lipid producing microalgal strains and high harvesting costs. The first problem is essentially that microalgae cannot grow rapidly and produce large amounts of lipids simultaneously. The second problem is that microalgae are microscopic organisms that are currently mostly harvested by centrifugation, a very expensive and energy-intensive step that is not easily scalable. Here we present a new approach that addresses both problems. UV-C radiation was used as an easily-applied external stimulant to rapidly induce lipids and also as a new method to induce overnight settling of microalgae for harvesting (a step that would normally require expensive flocculants or centrifuges).

In the first study conducted on *Chlorella* sp. BR2 with different dosages of UV-C radiation, maximum lipid fluorescence was measured after cultures were radiated with 500 mJ/cm^2. Moreover, the lipid induction was also reflected by an increase in total fatty acids after exposing to 100 and 250 mJ/cm^2 of UV-C radiation. One of the major findings in this study was that there was a significant increase in total unsaturated fatty acid when compared to total saturated fatty acids in the microalgae.

In the second phase of this study, to test whether nutrient starvation and UV-C treatment can lead to further lipid biosynthesis and facilitate settling in flagellate microalgae, combined sequential stress treatments were carried out on *Tetraselmis* sp. (M8). Maximum lipid induction was displayed by cultures radiated with 100 mJ/cm^2 and 250 mJ/cm^2 under laboratory conditions, and a dose of 48 J/cm^2 was found suitable for 12 cm-deep *Tetraselmis* 1000-L outdoor raceway pond cultures containing1.5x106 cells/mL. UV-C-induced settling occurred overnight within 10 h. The procedure essentially separates biomass growth from lipid accumulation and harvesting, and was optimized to be completed within 48 h for *Tetraselmis* sp (M8) using pilot-scale outdoor cultivation. Interestingly, the decrease of saturated fatty acids (SFAs) like C18 and C20 corresponded to the increase of unsaturated fatty acids (USFAs) like C16:2, C16:4, C18:1, C18:2, C18:3 and C20:4; which also coincided with the results obtain from *Chlorella* sp. BR2. Moreover,

an increase in reactive oxygen species (ROS) was also reported followed by an up-regulation of enzymatic activity of superoxide dismutase and glutathione reductase as well as lipid peroxidation, indicating high oxidative damage.

Experiments using UV-C radiation on C*hlorella* sp. BR2 *and Tetraselmis* sp. M8 showed that this external stress can stimulate lipid accumulation, especially PUFAs. Hence, it was tested weather UV-C radiation could be used to induce high-value fatty acids in other algae, including the highly productive oleaginous microalga *Nannochloropsis* sp. BR2. In particular the production of eicosacenoid pentanoic acid (EPA), an important omega-3 fatty acid with proven health benefits, was targeted. Optimised combined sequential stress treatments were carried out to achieve this aim. Comparing different USFAs, cultures treated with 100 and 250 mJ/cm^2 UV-C showed significant increases for all detected USFAs, most notably C20:5 (EPA) which accounted to be nearly 50 % of the total fatty acid, and was increased highly significantly after exposure to 100 mJ/cm^2 (P=0.0075) followed by 250 mJ/cm^2 (P= 0.0308).

From the above studies, it was discovered that short exposure of UV-C radiation could significantly induce lipid biosynthesis, most importantly polyunsaturated fatty acids (PUFAs) and cause oxidative damage. As carotenoids have been proven to have high anti-oxidative activity and scavenge free radicals the hypothesis was to use a small dosage of UV-C radiation to induce carotenoids in *Dunaliella salina* and *Haematococcus* sp., two microalgae that were found to contain high levels of beta-carotene and astaxanthin, respectively. A significant increase in total carotenoids and beta-carotene was observed after exposure to 50 mJ/cm^2 for *D.salina* and two-fold increase in total astaxanthin production was observed at 30 mJ/cm^2 for *Haematococcus* sp. Whereas, radiation of 50 mJ/cm^2 was found sufficient to detach flagellae in both species tested and approximately 95% of settling was observed after 15 h and 3 h for *D.salina* and *Haematococcus* sp., respectively. This was the first study to report UV-C radiation not only induces lipids biosynthesis, but also induces carotenoids biosynthesis in *D.salina* and *Haematococcus* sp.

Future studies are well-positioned to take the findings of this thesis to commercial levels of large-scale production of microalgae, where the use of UV-C could assist in lipid biosynthesis, in particular high-value fatty acids, such as EPA, carotenoid biosynthesis and settling of flagellate microalgae. During the present study, a 250,000-L algae demonstration farm was constructed in Pinjarra Hills adjacent to the Brisbane River to

develop large-scale production. Preliminary tests showed that UV-C exposure in pipes maybe the best option for cost-effective induction of lipids and/or carotenoids and settling for microalgal biomass harvesting.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Table of Contents

Acknowledgements xii

List of Abbreviations xxii

Project background and Research questions 1

Aims of the study 3

References 4

Chapter 1:High Lipid Induction in Microalgae for Biodiesel Production 5

1. Introduction 6

2. Lipids in Microalgae 7

3. Methods of Lipid Induction 8

3.1. Nutrient Starvation 10

3.2. Temperature Stress 14

3.3. Salinity-Induced Lipid Production 16

3.4. The Effect of pH and Heavy Metals Stress 17

3.5. Light Irradiation Stress 19

3.6. UV Irradiance for Lipid Induction 21

5. Conclusions and Future Directions 25

References 25

Chapter 2:Critical analysis of current microalgae dewatering techniques 35

Introduction 36

Primary harvesting 37

Secondary dewatering 44

Techno-economic assessment 46

Classification of current harvesting processes 48

Bio-chemically-based methods 51

Emerging technologies 51

Conclusion 52

Future perspective.....53
Executive summary.....53
References.....54
Chapter 3 - Rapid lipid induction in *Chlorella* sp. by UV-C radiation.....61
Overview.....61
Key Findings.....61
Chapter 3 - Rapid lipid induction in *Chlorella* sp. by UV-C radiation (Submitted).....62
Abstract.....62
Introduction.....62
Materials and methods.....64
Microalgae cultivation and UV-C treatment.....64
Lipid fluorescence analysis and GC-MS analysis.....65
Microscopic analyses.....66
Analytical methods.....66
Results.....66
UV-C–irradiation stimulates microalgal lipid accumulation but increases cell mortality.....66
UV-C induces lipid fluorescence in *Chlorella* populations.....67
.....**Error! Bookmark not defined.**
UV-C treatment induces production of polyunsaturated fatty acids (PUFA).....70
Discussion.....71
References.....78
Chapter 4.....82
Overview.....82
Key Findings.....82
Chapter 4:UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production.....83

Abstract 83
Introduction 84
Methods 85
Enzymatic assays 87
Results 90
Discussion 101
Acknowledgements 103
References 103
Chapter 5: 118
Overview 118
Key Findings 118
Chapter 5: Rapid induction of omega-3 fatty acids (EPA) in *Nannochloropsis* sp. by UV-C radiation (Submitted) 119
Abstract 119
Introduction 119
Materials and methods 121
Lipid fluorescence analysis and GC-MS analyses 121
Results 122
Fatty acid profiling in *Nannochloropsis* following UV-C radiation shows lipid induction and a shift towards LC-PUFAs 128
Discussion 130
References 136
Chapter 6: 139
Overview 139
Key Findings 139
Chapter 6: UV-C mediated rapid carotenoid induction and enhanced harvesting performance of *Dunaliella salina* and Haematococcus *pluvialis* (Submitted) 140

Abstract 140
Introduction 140
Materials and methods 143
Results 146
Discussion 154
References 157
Conclusion and Future direction 161
Future directions 163
Large-scale optimisation of LIS treatment at demonstration facility, Pinjarra Hills UQ. 163
Gene expression analysis and transformation. 164
Optimisation of UV-C settling technique 164
UV-C for extraction of oil from microalgae 164
Optimisation of UV-C method for other microalgal species 165
Appendices 166

List of Figures

Chapter 1: High Lipid Induction in Microalgae for Biodiesel Production

Figure 1**.** Lipid induction in microalgae under stress condition.....9

Chapter 2 - Critical analysis of current microalgae dewatering techniques

Figure 1- Overview of microalgae harvesting techniques 40

Figure 2- Interaction between different harvesting techniques.....49

Chapter 3 - Rapid lipid induction in Chlorella sp. by UV-C radiation

Figure 1 Nile red-stained *Chlorella* sp. BR2 cells observed at 40x magnification with a scale size of 50 µm. 67

Figure 2: FACS analysis of *Chlorella* sp. BR2 at 24 h after receiving different UV-C dosages. 69

Figure 3: Total fatty acids produced by different UV-C-treated *Chlorella* sp. BR2 cultures. Values are mean ± SE from three separately grown cultures (n = 3); bars with different letters indicate significant differences (*P*<0.05).....70

Figure 4: Effect of low dosage of UV-C radiation on the fatty acid synthesis pathway. Energy obtained from UV-C radiation might help in conversion of saturated fatty acids that serve as a storage function in plastids to unsaturated fatty acids that serve as antioxidants and repair damage to membrane lipids.....72

Online Resource Figure 1: Analysis of the non-polar fraction of TAGs in C*hlorella* sp. BR2 treated with different UV-C radiation doses (0-1000 mJ/cm^2). 74

Online Resource Figure 10: FACS analysis of *Chlorella* sp. BR2. Shown are cells without Nile red staining (Unstained Cells) and Nile red-stained with different UV-C dosages ranging from 0 mJ/cm^2 (Control) to 1000 mJ/cm^2 showing P1 and P2 populations. The Y-axis shows fluorescence intensity at the phycoerythrin excitation wavelength of 575 nm and the X-axis shows the forward scatter based on cell size. 75

Online Resource Figure 3: Comparison of different saturated fatty acids present in *Chlorella* sp. BR2 cultures treated with different doses of UV-C radiation. Values are mean ± SE from three separately-grown cultures (n = 3); bars with different letters indicate significant differences (*P*<0.05).....76

Online Resource Figure 4: Comparison of different unsaturated fatty acids present in *Chlorella* sp. BR2 cultures treated with different doses of UV-C radiation. Values are mean ± SE from three separately-grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).....77

Chapter 4 - UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production

Figure 1. Nile red-stained cells of *Tetraselmis* sp. M8 that received different doses of UV-C exposure. 91

Figure 2. Lipid induction in *Tetraselmis* sp. M8 cultures at 24 h after receiving different UV-C dosages.....93

Figure 3. Settling of control and UV-C-treated algal culture in 1000-L airlift-mixed raceway ponds. 95

Figure 4. Lipid fluorescence of Nile red-stained cells in control and UV-C-treated raceway ponds. 97

Figure 5. Biomass dry weights and fatty acid profiles in harvested culture of control and UV-C-treated raceway-grown algal cultures. 98

Chapter 5: Rapid induction of omega-3 fatty acids (EPA) in *Nannochloropsis sp*. by UV-C radiation

Figure 1: Nile red-stained cells of *Nannochloropsis* sp. BR2 that received different doses of UV-C exposure after nutrient starvation. 124

Figure 2: FACS analysis of *Nannochloropsis* sp. BR2 unstained cells (a), Nile red-stained mock-treated control cells (b), UV-C treated cells (c-f) at 100, 250, 500 and 1000 mJ/cm^2, respectively.....126

Figure 3: Lipid induction in *Nannochloropsis* sp. BR2 at 24 h after receiving different UV-C dosages.FACS analysis of Nile red-stained cells showing distribution (a) and lipid fluorescence (b) of low (P1) and high (P2) fluorescence cell populations and of the total population (c). 127

Figure 4: Fatty acid profiling and quantification by GC-MS showing (a-c) total fatty acids as well as saturated (SFA) and unsaturated (USFA) fatty acids, respectively, produced by different UV-C-treated *Nannochloropsis* sp. BR2 cultures. 129

Figure 5: Dry weight and EPA proportion changes in *Nannochloropsis* sp. BR2, following nutrient starvation and different UV-C exposures. 130

Supplementary Fig. 1: Comparison of different saturated fatty acids present in *Nannochloropsis* sp. BR2 following different treatments of UV-C radiation. 134

Supplementary Fig. 2: Comparison of different unsaturated fatty acids present in *Nannochloropsis* sp. BR2 following different treatments of UV-C radiation. 135

Chapter 6: UV-C mediated rapid carotenoid induction and enhanced harvesting performance of Dunaliella salina and Haematococcus pluviali.

Figure 1: a) Kill curve of *D. salina and H. pluvialis* cells, showing the number of survival cells after different UV-C radiation dosages; b) Settling curve of *D. salina* and *H*. *pluvialis*. 148

Figure 2: a-c) Nile red-stained cells of *D. salina* cultures exposed to UV-C radiation at 0 (control), 75 and 100 mJ/cm^2, respectively. d-f) Nile red-stained cells of *H. pluvialis* cultures exposed to UV-C radiation at 0 (control), 30 and 100 mJ/cm^2, respectively. g,h) Lipid fluorescence analysis of different UV-C treated samples of *D. salina* and *H. pluvialis*. 149

Figure 3:a-c) Total carotenoids, β-carotenoids and lutein measured in *D. salina* cells following different UV-C treatments.. 151

Figure 4: a-c) Visible carotenoid induction in UV-C-treated *H. pluvialis* cultures at 0 (control), 30 and 100 mJ/cm^2, respectively. d-g) Total astaxanthin, cis-astaxanthin, mono-astaxanthin and di-astaxanthin contents in *H. pluvialis* cells upon different UV-C radiations. 152

Figure 5:a-c) Total fatty acids, total unsaturated fatty acids and total saturated fatty acids induced in *D. salina* cultures at different dosages of UV-C radiation. ……153

Figure 6: Decomposition of unstable peroxides (TBRAS) assay of UV-C-stressed and mock-treated *H. pluvialis* cells at different dosages. 154

List of Tables

Chapter 1: High Lipid Induction in Microalgae for Biodiesel Production

Table 1**:** Examples of different types of nutrient starvation stress which have been studied to induce lipids in microalgae. 12

Table 2**:** Lipid induction in microalgae with different temperatures. 16

Table 3**:** Examples of lipid induction in microalgae due to salinity and pH stress. 19

Table 4**:** Lipid induction due to different light irradiation stress in microalgae. 23

Chapter 2 - Critical analysis of current microalgae dewatering techniques

Table 1:Examples of various flocculation studies that have been used to harvest microalgae 41

Table 2 :Examples of various flotation studies that have been used to harvest microalgae. 43

Table 3:Examples of various filtration studies that have been used to harvest microalgae 46

Table 4:Cost of harvesting 10,000 L of *Chlorella* sp. with different harvesting techniques 47

Chapter 4 - UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production.

Table 1. Costs of harvesting 10,000 L of *Tetraselmis* sp. culture with different harvesting techniques. 100

Conclusion and Future direction

Table 1- Summary of different microalgae tested by UV-C lipid induction and settling (LIS) method. Red colour text indicates that the method has been optimised for pilot scale. ... 163

List of Abbreviations

FAME	Fatty acid methyl ester
CO_2	Carbon dioxide
UV-C	Ultraviolet-C
FA	Fatty Acid
PUFA's	Ploy Unsaturated Fatty acids
SFA's	Saturated Fatty Acids
NADPH	Nicotinamide adenine dinucleotide phosphate
TAG	Triacylglycerol
HRP	High rate pond
PBR	Photobioreactor
GHG	Greenhouse gas
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NaCl	Sodium chloride
HCl	Hydrochloric acid
TSS	Total suspended solids
ECF	Electrocoagulation flocculation
M	Molar
mM	Mill molar
Kg	Kilogram
g	Gram
mg	Milligram
µg	Microgram
L	Litre
µL	Microliter
kWh	Kilowatts hour

nm	Nanometre
µm	Micrometre
cm	Centimetre
m^3	Cubic metre
K'	Growth rate
ppm	Parts per million
ppt	Parts per thousand
rpm	Rounds per minute
min	Minutes
s	Seconds

Project background and Research questions

Sustainable production of renewable energy is being debated globally since it is increasingly understood that first generation biofuels, primarily produced from food crops and mostly oil seeds, compete for arable land, freshwater or bio-diverse natural landscapes and are limited in their ability to achieve targets for biofuel production. These concerns have increased the interest in developing second and third generation biofuels such as lignocellulosics and microalgae, respectively, which potentially offer great opportunities in the longer term and do not need to compete for arable land and precious freshwater [1,2]. Microalgae have thus become increasingly important feedstocks for different industrial processing applications. Triacylglycerides (TAGs) generally serve as energy storage in microalgae that, once extracted, can be easily converted into biodiesel through transesterification reactions [3,4]. These neutral lipids bear a common structure of triple esters where usually three long-chain fatty acids (FAs) are coupled to a glycerol molecule. Transesterification displaces glycerol with small alcohols (e.g., methanol). Recently, the rise in petroleum prices and the need to reduce greenhouse gas emission has seen a renewed interest in large-scale biodiesel production [5].

Within the last few decades the concept of lipid induction in microalgae has been intensively studied to increase TAG production in microalgae, but at present, different lipid induction techniques have not been compared to each other. Here we provide a review of different lipid inducing techniques in microalgae and their potential to be used for biodiesel production (Chapter 1: Sharma, K.K.; Schuhmann, H.; Schenk, P.M. High Lipid Induction in Microalgae for Biodiesel Production. *Energies* 2012, *5*,1532-1553.). It is clear that amongst different lipid induction techniques, nitrate starvation is most widely applied and studied in almost all the microalgae species that can be used in the commercial production of bio-fuel. The possible reason for this would be it is easy to apply nitrate stress on microalgae by just subtracting the nitrate source in the growth media or letting the culture starve and thus trigger nitrate stress. However, it still takes 3-5 days until significant amounts of lipids are induced which is complemented with slow growth rates and low cell counts and thus finally affects the total biomass (Widjaja *et al.* 2009). Change in temperature, pH, salinity and heavy metals can also induce lipids, but it is difficult to regulate these on large scale hence is inappropriate in commercial production of biofuel. Genetically modified strains of microalgae have the potential to produce more lipids, but there are regulatory issues to use these strains in outdoors cultivation, thus increasing cost.

Microalgae are typically 2-50 microns in size with a negative charge on the cell surface [6-8], but some microalgae, under certain conditions, have a larger cell size. In most cases they are motile, i.e. swimming or gliding such as dinoflagellates or raphid diatoms and form stable suspensions. Unfortunately, microalgal biomass is fairly dilute in cultures (up to 0.3-0.5 g dry biomass per liter), resulting in difficulties to harvest and dewater algae cost-effectively [6]. Microalgae harvesting can typically make up to 20–30% of the total biomass production cost [8-11]. This makes the harvesting process a major bottleneck, hindering the development of the microalgae industry. To date, there are a multitude of techniques being used for microalgae dewatering, but with low economical feasibility and are based on their large biodiversity, microalgae harvesting processes are to a large extent species-specific, (Chapter 2 : Sharma KK, Grag S, Li Y, Schenk PM "Critical analysis of microalgae harvesting technology". Biofuels, 2013).

Thus there is need of a superior technique that can not only induce lipids in exponential phase within hours, but also bridge the gap between the time taken in microalgae biomass production to harvesting the biomass. After considering different lipid induction (Chapter 1) and harvesting (Chapter 2) techniques it was concluded that, lipid induction with light irradiation is a potential method to serve this purpose.

Light irradiation from the sun can be divided into visible light (740 nm-380 nm) and carries energy of 3.10 eV/photon. Ultraviolet light consists of UV-A (400 nm-315 nm) with an energy range of 3.10–3.94 eV/photon, UV-B (315 nm-280 nm) with an energy range of 3.94–4.43 eV/photon, and UV-C (280 nm-100 nm) with an energy range of 4.43–12.4 eV/photon. UV-C light never reaches the Earth's surface as it is deflected back from the ozone layer due to its shorter wavelength. UV-A and UV-B have already been tested and proven to be successful to induce lipids in microalgae, however the time taken for lipid induction was more than 2-3 days. As UV-C light has the maximum amount of energy per photon, our hypothesis was to use UV-C (253 nm) radiation as a stress induction technique after reaching the maximum biomass (cell count), so as to minimize the biomass loss and obtain high lipid productivity within 24 h.

Aims of the study

I. **Develop a method for rapid induction of lipids in microalgae.**

- Evaluate and optimise rapid induction of lipids in freshwater *Chlorella* sp. BR2 microalgae by UV-C radiation.
- Evaluate and optimise rapid induction of lipids EPA in the marine alga *Nannochloropsis* sp. BR2.
- Evaluate and optimise rapid induction of lipids in the marine flagellate alga *Tetraselmis* sp. M8 by UV-C radiation.
- Increase our understanding of UV-C-mediated stress for lipid biosynthesis in microalgae

II. **To develop a new technique for effective harvesting of microalgae method of Lipid induction and Settling (LIS) in microalgae (Pilot scale)**.

- Develop a method to simultaneously induce lipids and settling in *Tetraselmis* sp. M8.
- Apply Lipid induction and Settling (LIS) in microalgae at pilot scale.

III. **To develop and optimise methods of carotenoid/astaxanthin induction and harvesting by settling in *D. salina* and *Haematoccocus* sp.**

- Develop and optimise carotenoid and fatty acid induction, as well as settling in *D. salina* by UV-C radiation.
- Develop and optimise astaxanthin and harvesting by settling in *Haematococcus* sp. by UV-C radiation.
- Increase our understanding of UV-C-mediated stress for carotenoid biosynthesis in microalgae

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Chapter 1: High Lipid Induction in Microalgae for Biodiesel Production

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Abstract: Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. High lipid productivity of dominant, fast-growing algae is a major prerequisite for commercial production of microalgal oil-derived biodiesel. However, under optimal growth conditions, large amounts of algal biomass are produced, but with relatively low lipid contents, while species with high lipid contents are typically slow growing. Major advances in this area can be made through the induction of lipid biosynthesis, e.g., by environmental stresses. Lipids, in the form of triacylglycerides typically provide a storage function in the cell that enables microalgae to endure adverse environmental conditions. Essentially algal biomass and triacylglycerides compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis. There has been a wide range of studies carried out to identify and develop efficient lipid induction techniques in microalgae such as nutrients stress (e.g., nitrogen and/or phosphorus starvation), osmotic stress, radiation, pH, temperature, heavy metals and other chemicals. In addition,

several genetic strategies for increased triacylglycerides production and inducibility are currently being developed. In this review, we discuss the potential of lipid induction techniques in microalgae and also their application at commercial scale for the production of biodiesel.

Keywords: algaculture; biofuels; biodiesel; induction; lipids; microalgae; oil production; triacylglycerides

1. Introduction

Sustainable production of renewable energy is being debated globally since it is increasingly understood that first generation biofuels, primarily produced from food crops and mostly oil seeds, compete for arable land, freshwater or biodiverse natural landscapes and are limited in their ability to achieve targets for biofuel production. These concerns have increased the interest in developing second and third generation biofuels such as lignocellulosics and microalgae, respectively, which potentially offer great opportunities in the longer term and do not need to compete for arable land and precious freshwater [1,2]. Due to continuous and increasing combustion of fossil carbon, the amount of greenhouse gas CO_2 has increased. As a result global warming and climate change are threatening ecological stability, food security and social welfare [3,4]. The transportation and energy sector are the two major sources, responsible for the generation of 20% and 60% of greenhouse gases (GHG) emissions, respectively, and it is expected that with the development of emerging economies the global consumption of energy will rise considerably and this will lead to more environmental damage [5].

Photosynthesis is the only process that can convert CO_2 into organic compounds with high energy content, and thus can provide a source for sustainable transport fuel production. There is an urgent need to develop technologies which are able to produce an additional five to six billion tons of organic carbon apart from the current harvest from agricultural crops [3]. Large-scale cultivation of microalgae may be 10–20 times more productive on a per hectare basis than other biofuel crops, are able to use a wide variety of water sources, and have a strong potential to produce biofuels without the competition for food production [2]. Algae can be produced either as macrophytes via marine aquaculture [6] or in large-scale microalgae cultivation systems in open ponds or in photobioreactors [1]. Microalgae are currently considered the most promising types of

algae for biofuel production, based on their high lipid contents. Recent progress in the production of microalgae has been intensively reviewed [7], and future perspectives have been presented by Stephens *et al*. [5]. Microalgae can also be cultivated as an integrated concept with wastewater treatment to optimize the energetic and financial input for the production process [8].

Triacylglycerides (TAGs) generally serve as energy storage in microalgae that, once extracted, can be easily converted into biodiesel through transesterification reactions [3,9]. These neutral lipids bear a common structure of triple esters where usually three long-chain fatty acids (FAs) are coupled to a glycerol molecule. Transesterification displaces glycerol with small alcohols (e.g., methanol). Recently, the rise in petroleum prices and the need to reduce greenhouse gas emission has seen a renewed interest in large-scale biodiesel production [10].

Within the last few decades the concept of lipid induction in microalgae has been intensively studied to increase TAG production in microalgae, but at present different lipid induction techniques have not been compared to each other. Here we provide a review of different lipid inducing techniques in microalgae and their potential to be used for biodiesel production.

2. Lipids in Microalgae

Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. Lipids produced by microalgae can be grouped into two categories, storage lipids (non-polar lipids) and structural lipids (polar lipids). Storage lipids are mainly in the form of TAG made of predominately saturated FAs and some unsaturated FAs which can be transesterified to produce biodiesel. Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs), which are also essential nutrients for aquatic animals and humans. Polar lipids (phospholipids) and sterols are important structural components of cell membranes which act as a selective permeable barrier for cells and organelles. These lipids maintain specific membrane functions, providing the matrix for a wide variety of metabolic processes and participate directly in membrane fusion events. In addition to a structural function, some polar lipids may act as key intermediates (or precursors of intermediates) in

cell signaling pathways (e.g., inositol lipids, sphingolipids, oxidative products) and play a role in responding to changes in the environment.

Of the non-polar lipids, TAGs are abundant storage products, which can be easily catabolized to provide metabolic energy [11]. In general, TAGs are mostly synthesized in the light, stored in cytosolic lipid bodies, and then reutilized for polar lipid synthesis in the dark [12]. Microalgal TAGs are generally characterized by both, saturated and monounsaturated FAs. However, some oil-rich species have demonstrated a capacity to accumulate high levels of long-chain polyunsaturated fatty acids (PUFA) as TAG [13,14]. A detailed study on both accumulation of TAG in the green microalga *Parietochloris incisa* and storage into chloroplastic lipids (following recovery from nitrogen starvation) led to the conclusion that TAGs may play an additional role beyond being an energy storage product in this alga [13,15]. Hence, PUFA-rich TAGs are metabolically active and are suggested to act as a reservoir for specific fatty acids. In response to a sudden change in the environmental condition, when the *de novo* synthesis of PUFA may be slower, PUFA-rich TAG may donate specific acyl groups to monogalactosyldiacylglycerol (MGDG) and other polar lipids to enable rapid adaptive membrane reorganization [15,16].

3. Methods of Lipid Induction

The ability of microalgae to survive in diverse and extreme conditions is reflected in the tremendous diversity and sometimes unusual pattern of cellular lipids obtained from these microalgae [17]. Moreover, some of these microalgae can also modify lipid metabolism efficiently in response to changes in environmental conditions [12,18]. A review of microalgal lipid metabolism has recently been published [19]. Under optimal growth conditions, large amounts of algal biomass are produced but with relatively low lipid contents (Figure 1), which constitute about 5–20% of their dry cell weight (DCW), including glycerol-based membrane lipids. Essentially, microalgae biomass and TAGs compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis. Under unfavorable environmental or stress conditions many microalgae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20–50% DCW), mainly in the form of TAG, enabling microalgae to endure these adverse conditions (Figure 1).

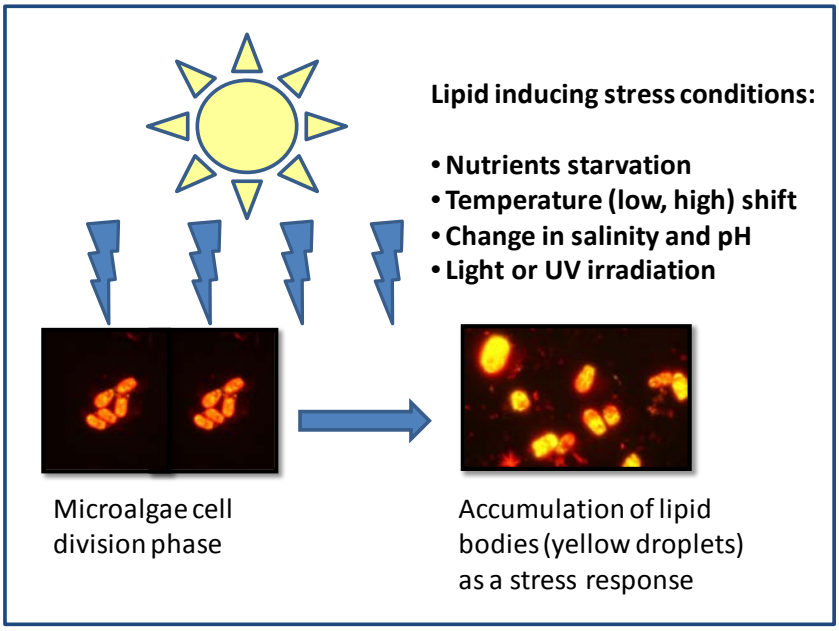

Figure 1**.** Lipid induction in microalgae under stress condition.

High capital costs due to low lipid productivity of FA-synthesizing microalgae are a major bottleneck, hindering the commercial production of microalgal oil-derived biodiesel. One who has grown microalgae under laboratory or outdoor condition is well aware of the fact that to obtain high lipid content, external stress or lipid induction techniques need to be applied. Many microalgae produce saturated and unsaturated FAs naturally under ideal growth conditions, which have high nutritional value, but are less ideal for biofuels. However, the synthesis of neutral lipids in the form of TAG can be induced in many species under stress conditions, and these lipids are suitable precursors for biodiesel production [20,21]. The occurrence and the extent to which TAGs are produced is species/strain-specific, and are ultimately controlled by the genetic make-up of individual organisms. Synthesis and accumulation of large amounts of TAGs accompanied by considerable alterations in lipid and FA composition can occur in microalgae when placed under stress conditions imposed by chemical or physical environmental stimuli, either acting individually or in combination [21]. There has been a wide range of studies carried out on lipid induction techniques in microalgae such as the use of nutrients stress, including nitrogen and/or phosphorus starvation, light irradiation, pH, temperature, heavy metals and other chemicals. The following paragraphs review the different TAG induction techniques and discuss their potential in different microalgae species.

3.1. Nutrient Starvation

Nutrient availability has a significant impact on growth and propagation of microalgae and broad effects on their lipid and FA composition. Environmental stress condition when nutrients are limited, invariably cause a steadily declining cell division rate. Surprisingly, active biosynthesis of fatty acids is maintained in some algae species under such conditions, provided there is enough light and CO_2 available for photosynthesis [12]. When algal growth (as measured by cell divisions) slows down and there is no requirement for the synthesis of new membrane compounds, the cells instead divert and deposit fatty acids into TAG. Under these conditions, TAG production might serve as a protective mechanism. Under normal growth conditions, ATP and NADPH produced by photosynthesis are consumed by generating biomass, with ADP and $NADP^+$ eventually being available again as acceptor molecules in photosynthesis. When cell growth and proliferation is impaired due to the lack of nutrients, the pool of the major electron acceptor for photosynthesis, $NADP^+$, can become depleted. Since photosynthesis is mainly controlled by the abundance of light, and cannot be shut down completely, this can lead to a potentially dangerous situation for the cell, damaging cell components. NADPH is consumed in FA biosynthesis, therefore, increased FAs production (which in turn are stored in TAGs) replenishes the pool of $NADP^+$ under growth-limiting conditions [12,21].

Nutrient starvation is one of the most widely used and applied lipid induction techniques in microalgal TAG production and has been reported for many species (Table 1). For example, when the diatom *Stephanodiscus minutulus* was grown under silicon, nitrogen or phosphorus limitation, an increase in TAG accumulation and a decrease of polar lipids (as percentage of total lipids) was noticed in all of the nutrient-limited cultures [22]. In the green alga *Chlamydomonas moewusii*, nutrient limitation resulted in decreased PUFA C16:3, C16:4, and C18:3 contents whereas overall levels of C16:1 and C18:1 FA were increased [23].

Nitrogen is the single most critical nutrient affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly TAG, in response to nitrogen deficiency has been observed in numerous species or strains of various microalgae [24–26]. Hu *et al.* [27] conducted a study on nitrogen stress responses of several green microalgae, diatoms and cyanobacteria and all tested species showed a significant rise in lipid production. A detailed and large-scale model of lipid induction by nutrient starvation (nitrogen, phosphorus) on several diatoms, green algae, red algae, prymnesiophytes and

eustimatophytes is presented in a study carried out by Rodolfi *et al.* [28]. In the diatom *Cyclotella cryptica,* higher levels of neutral lipids (primarily TAG) and higher proportions of saturated and mono-unsaturated FAs were produced due to silicon deficiency [20]. However, only a small increase in TAG levels (from 69 to 75% from total lipids) together with phospholipids (from 6 to 8%) was reported for the microalga *Phaeodactylum tricornutum* as a result of reduced nitrogen concentrations [29]. *Scenedesmus* sp. subjected to nitrogen or phosphorus limitation showed an increase in lipids as high as 30% and 53%, respectively [30]. Lipid content of freshwater green alga *Chlorella vulgaris* could be significantly increased by 40% in low nitrogen-containing medium [31]. With manipulated culture conditions of 1 mM KNO_3, 1.0% CO_2, 60 µmol photon m^{-2} s^{-1} and 25 °C, lipid production of *C. vulgaris* was increased by 2.5-fold [32]. In addition, lipid stimulation in *Chlorella* was also achieved via silicon deficiency [33] and iron supplementation [34]. Moreover, it was found for *C. vulgaris* that changing from normal nutrient to nitrogen depletion media gradually changed the lipid composition from free FA-rich lipids to lipid mostly contained as TAG [35]. Nitrogen starvation in microalgae not only affects the fatty acid metabolism, but also affects pigment composition. For *Parietochloris incise* grown in nitrogen-replete medium a considerable increase in the ratio of carotenoid and chlorophyll contents was recorded [36].

Phosphorus limitation resulted in increased lipid content, mainly as TAG, in *P. tricornutum, Chaetoceros* sp*., Isochrysis galbana* and *Pavlova lutheri*, but decreased lipid content in *Nannochloris atomus* and *Tetraselmis* sp*.* [37]. Due to phosphorus deprivation, production of C16:0 and C18:1 was increased and production of C18:4ω3, C20:5ω3 and C22:6ω3 was decreased [37]. In contrast, for phosphorus-starved cells of the green alga *Chlorella kessleri*, an elevated level of unsaturated fatty acids in all identified individual lipids, namely phosphatidylcholine (PC), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), andsulfoquinovosyl diacylglycerols (SQDG) were found [38]. Phosphorus limitation was also found to increase the overall TAG production from 6.5% up to 39.3% with a gradual decrease in eicosapentaenoic acid (EPA) concentration. The cellular total lipid content increased, mainly due to TAG accumulation in *Monodus subterraneus* [15]. In studies carried out on other organisms, including higher plants, the authors have also acknowledged replacement of membrane phospholipids by non-phosphorus containing glycolipids and betain lipids under phosphate limitation [39,40].

A study by Sato *et al.* [17] on sulphur and phosphorus depletion in green alga *C. reinhardtii* showed that sulphur depletion leads to decrease in SQDG but on the other hand PG was increased by 2-fold, representing a compensatory mechanism where lipids containing sulphur are substituted by lipids containing phosphate. When *C. reinhardtii* was grown in media with limited phosphorus it showed a 40% decrease in PG and also stimulated increase in the SQDG content. Thus, mechanisms that keep the total sum of SQDG and PG concentrations constant under both phosphorus and sulphur-limiting conditions appear to occur [17]. Other studies have also shown that sulfur deprivation led to increased total lipid content in the green algae *Chlorella* sp. and *C. reinhardtii* [41].

Based on the literature reviewed, it is clear that amongst all nutrient starvation approaches, nitrogen starvation technique is most widely applied and studied in almost all the microalgae species that can be considered for the production of biofuel (Table 1). Nitrogen is the most growth-limiting factor for eukaryotic microalgae and would be one of the first nutrients to be depleted during algae cultivation. It is relatively easy to apply controlled nitrogen stress on microalgae by subtracting the nitrogen source in the growth media. Moreover all the microalgae species studied so far (Table 1), seem to increase TAG production under nitrogen stress. Hence, nitrogen starvation is the most successful lipid inducing technique at present. However, high lipid production due to nitrogen stress may take 2–5 days and is complemented with slow growth rates and low cell counts and thus finally effecting the total biomass and lipid productivity as detailed by Widjaja *et al.* [35].

Table 1**.** Examples of different types of nutrient starvation stress which have been studied to induce lipids in microalgae.

Microalgae species or strain	Nutrient stress	Changes in lipid profile after induction	Reference
Chlamydomonas reinhardtii, Scenedesmus subspicatus	N limitation	Increase in total lipids (lipid: amide ratio)	[42]
Nannochloropsis oculata	N limitation	Total lipid increased by 15.31%	[43]
Chlorella vulgaris	N limitation	Total lipid increased by 16.41%	[43]
Chlorella vulgaris	N limitation	Lipid productivity of 78 mg/L d	[24]
***Chlorella* sp.**	N limitation	Lipid productivity of 53.96 ± 0.63 mg/L d	[25]
Phaeodactylum tricornutum	N limitation	TAG levels increased from 69 to 75%	[29]

Table 1. *Cont.*

Dunaliella tertiolecta	N limitation	Five times increase in lipid fluorescence	[44]
Chlorella vulgaris	Nitrogen medium	Lipids increased by 40%	[31]
Chlorella vulgaris	Nlimitation	Increase in TAG	[35]
***Chlorella* sp.**	Nutrient-deprived conditions (nitrogen, phosphate-potassium, iron, and all three combined)	Total lipid production of 49.16 ± 1.36 mg/L d	[25]
***Chlorella* sp.**	Urea limitation	Total lipid productivity of 0.124 g/ L d	[26]
Neochloris oleoabundans	Ammonium nitrate	Lipid productivity of 0.133 g /L d	[45]
***Scenedesmus* sp.,** ***Coelastrella* sp.**	Combined effect of Ph and N-limitation	Increase in TAG	[46]
Phaeodactylum tricornutum* ,** ***Chaetoceros* sp.,** ***Isochrysis galbana	Phosphorus limitation	Increase in total lipids with higher relative content of 16:0 and 18:1	[37]
Monodus subterraneus	Phosphorus limitation	Increase in TAG	[15]
***Scenedesmus* sp**	Nitrogen and phosphorus starvation	Lipids increased 30% and 53%, respectively	[30]
***Chlorella* sp.**	Silicon deficiency	-	[33]
Chlorella kessleri	Phosphorus limitation	Increase in unsaturated FAs	[38]
Chlamydomonas reinhardtii	Sulphur limitation	PG was increased by 2-fold	[17]
Chlamydomonas reinhardtii	Sulphur limition	Increase in TAG	[41]
Cyclotella cryptica	Silicon starvation	Increased in total lipids from 27.6% to 54.1%	[47]

3.2. Temperature Stress

Temperature has been found to have a major effect on the fatty acid composition of microalgae (Table 2) [18,48]. A general trend towards increasing FA unsaturation with decreasing temperature and increasing saturated FA with increasing temperature has been observed in many microalgae and cyanobacteria (Table 2) [49–52]. It is generally accepted that many of the lipid profile changes alter the physical properties of membranes so that normal functions (e.g., ion permeability, photosynthetic and respiratory processes) can continue unimpaired [53]. The most commonly observed change in membrane lipids following a temperature shift is an alteration in FA unsaturation [54]. Due to their geometry, FAs with carbon-carbon double bonds cannot be as densely packed as saturated FA, therefore the fluidity of membranes containing unsaturated FA is increased. As membrane fluidity is decreased at lower temperatures, increased FA unsaturation provides an adaptation to the changing environment.

Dunaliella salina has been extensively analyzed for low temperature modification of lipid composition [12]. A temperature shift from 30 °C to 12 °C increased the level of unsaturated lipids significantly by 20% [12]. In *Ochromonas danica*, as the incubation temperature rose from 15 to 30 °C, the cell number per unit volume of medium was increased thus increasing total lipid content [55]. In *Chlorella vulgaris* and *Botryococcus braunii*, increased temperature resulted in a decrease of the relative content of unsaturated intracellular fatty FAs [56]. Increases in growth rate and total lipid production were obtained in *Nannochloropsis salina* with an increase in temperature [57]. Whereas, a decrease in culture temperature from 25 to 10 °C led to an elevation in the relative proportion of oleate but a decrease in linoleate and stearidonic acid (C18:4n-3) in the green alga *Selenastrum capricornutum* [58]. In a culture of *I. galbana* grown at 30 °C, total lipids accumulated at a higher rate with a slight decrease in the proportion of nonpolar lipids [59]. On the other hand, higher levels of omega-3 PUFA α-linolenic acid (ALA) and docosahexaenoic acid (DHA) with a corresponding decrease in saturated, monounsaturated, and linoleic fatty acids were found in the cells grown at 15 °C [59]. Moreover, in the diatom *P. tricornutum* the highest yields of PUFA and EPA per unit dry mass were 4.9 and 2.6%, respectively, when temperature was shifted from 25 °C to 10 °C for 12 h, with both being raised by 120% compared with the control [60].

Study on the effects of low temperatures in some higher plants have also been shown to increase the amount of unsaturated FAs [61]. Similar results were also obtained in

Chlorella ellipsoidea where the content of unsaturated FA was increased by 2-fold. Moreover, a low temperature-adapted strain of this species also showed increased ALA and, therefore, more unsaturation in its PG [62]. In the marine microalga *Pavlova lutheri,* significant changes in acidic lipid and fatty acid composition have been reported for cultures grown at 15 °C compared with 25 °C [63]. The culture grown at 15 °C resulted in an increased relative amount of EPA and DHA [63]. Variations of FA composition with growth temperature were also studied by Fork *et al.* on the thermophilic cyanobacterium *Synechococcus lividus* [64]. When the growth temperature was lowered from 55 °C to 38 °C, the amount of saturated FA C18:0 decreased while the unsaturated FAs C18:1 and C16:1 increased [64]. In general, there was an increase in the more fluid lipids in all of the lipid classes when the cells were grown at the lower temperature [64].

The cyanobacterium Sp*irulina platensis* and eukaryotic microalgae *Chlorella vulgaris* and *Botryococcus braunii* were studied for the effect of ambient temperature on the composition of intracellular FAs and the release of free fatty acids (FFA) into the medium [56]. It was found that all of the above species studied, regardless of their taxonomic status, responded to the temperature regime by similar changes in their intracellular FA composition: the relative content of more unsaturated FAs decreased and saturated FAs increased with the elevation of temperature [56].

In contrast, no significant change in the lipid content was observed in *Chlorella sorokiniana* grown at various temperatures [65]. There was no effect of temperature shift on the content of the acidic thylakoid lipids, SQDG and PG, in *C. reinhardtii* [17]. It should be noted that only a limited amount of information is available on this subject and that all studies were carried at laboratory scale where it is very easy to maintain the desired temperature. Thus maintaining, decreasing or increasing temperature is feasible only in closed system photobioreactors which are costly when compared to open systems. At present, we are not aware of any study that has highlighted the effect of temperature to induce lipids on large-scale cultivation; but as lipid profiles clearly change at different temperatures, properties of algal-derived biodiesel would also change for different climates and seasons. Different strains or species may be used for different seasons (e.g., summer or winter strains) and efforts are underway to use flue gases and other heat sources to increase algae growth in colder climates.

Table 2**.** Lipid induction in microalgae with different temperatures.

Microalgae species or strain	Stressing agent	Lipid profile change after induction	Reference
***Chaetoceros* sp.**	Grown at 25 °C	Total lipid increased by 16.8%	[49]
***Rhodomonas* sp.*, Cryptomonas* sp.*, Isochrysis* sp.**	Range of 27 °C to 30 °C	Lipid production increased by 15.5, 12.7, and 21.7% respectively	[49]
Nannochloropsis oculata	Increase from 20 °C to 25 °C	Lipid production increased by 14.92%	[43]
Isochrysis galbana	Increase from 15 °C to 30 °C	Increase in neutral lipids	[59]
Chlorella ellipsoidea	Lowering temperature	Unsaturated FA was increased by 2-fold	[62]
Nannochloropsis salina	Increase in temperature	Increase in total lipids	[57]
Dunaliella salina	Shift from 30 °C to 12 °C	Increase in unsaturated lipids	[12]
Ochromonas danica	Increase from 15 °C to 30 °C	Increase in total lipids	[55]
Selenastrum capricornutum	Temperature from 25 °C to 10 °C	Increase in oleate fatty acid	[58]
Isochrysis galbana	Grown at 30 °C	Increase in total lipids	[59]
Phaeodactylum tricornutum	Shifted from 25 °C to 10 °C for 12 h	Highest yields of PUFA and EPA	[60]
Pavlova lutheri	Grown at 15 °C	Increased relative amount of EPA	[63]
Spirulina platensis, Chlorella vulgaris, Botryococcus braunii	Increase in temperature	Saturated FAs increased	[56]

3.3. Salinity-Induced Lipid Production

Dunaliella species provide the best examples of microalgae that can tolerate high salt concentrations. The ability of *Dunaliella* species to proliferate over practically the saturation range of salinities makes them one of the favorite candidates to study salinity effects on microalgae [66–68]. In a study carried out by Azachi *et al.* [66] cells of *D. salina* were transferred from 0.5 to 3.5 M (29 to 205 g/L) NaCl, and there was a significantly higher ratio of C18 (mostly unsaturated) to C16 (mostly saturated) FAs in the cells grown

in 3.5 M (205 g/L) NaCl compared with those grown at 0.5 M (29 g/L) NaCl [66]. An increase of the initial NaCl concentration from 0.5 M (29 g/L) to 1.0 M (58 g/L) followed by further addition of NaCl to 2.0 M (117 g/L) during cultivation of *Dunaliella tertiolecta* resulted in an increase in intracellular lipid content and a higher percentage of TAG [67]. An even stronger increase in salinity from 0.4 M to 4 M (23 to 234 g/L) in *Dunaliella* sp. increased the proportion of total saturated fatty and monounsaturated fatty acids, whereas the proportion of PUFA was decreased [68].

The diatom *Nitzschia laevis* is known to produce high amounts of EPA [69]. When these cells were subjected to high salt concentrations, the degree of FA unsaturation of both neutral and polar lipid fractions increased sharply when salt concentrations increased from 10 to 20 g/L, but decreased at salt concentrations of 30 g/L [69]. Highest contents of total fatty acids, EPA and polar lipids were all obtained at NaCl concentration of 20 g/L, under which 71.3% of total EPA existed in polar lipid fractions [69]. The amount of total free sterols was also increased with an increase in salt concentration. In three marine heterotrophic microalgae strains, *Crythecodinium cohnii* ATCC 30556, *C. cohnii* ATCC 50051 and *C. cohnii* RJH grown at different salinities, the FA composition was also affected [70]. At 9 g/L NaCl, *C. cohnii* ATCC 30556 had the highest total FA content and DHA (C22:6) proportion. In contrast, *C. cohnii* ATCC 50051 and *C. cohnii* RJH had the highest DHA content at 5 g/L NaCl. *C. cohnii* ATCC 30556 and ATCC 50051 had the highest DHA yield (132 and 68 mg/L respectively) at 9 g/L NaCl while *C. cohnii* RJH had the highest DHA yield (129 mg/L) at 5 g/L NaCl [70]. Growth, lipid content and FA composition of heterotrophic microalga *Schizochytrium limacinum* OUC88 at different temperatures (16 °C, 23 °C, 30 °C and 37 °C) and salinities (0, 9, 18, 27 and 36 g/L) were analyzed [71]. Highest lipid content was obtained at salinities of 9–36 g/L at a temperature range of 16–30 °C and the content of saturated fatty acids C15:0 and C17:0 was increased greatly [71]. In addition, the ratio of DHA to DPA changed at different temperatures and salinities [71].

3.4. The Effect of pH and Heavy Metals Stress

Fluctuations of the pH in the medium also have been found to alter the lipid composition of microalgae (Table 3). For example, alkaline pH stress led to TAG accumulation in *Chlorella* CHLOR1 and was not dependent on nitrogen or carbon limitation levels, and led to a decrease in membrane lipids [72]. Based on morphological observations, alkaline pH inhibited the growth of microalgae, thus diverting the energy to form TAG [72]. The effects

of pH on the lipid and FA composition of a *Chlamydomonas* sp. isolated from a volcanic acidic lake, and *C. reinhardtii* have been studied and compared [73]. In the unidentified *Chlamydomonas* sp., FAs of polar lipids were more saturated than those in *C. reinhardtii*. The relative proportion of TAG (as percentage of total lipids) was higher in *Chlamydomonas* sp. grown at pH 1 than that in the cells cultivated at higher pH. The increase in saturation of fatty acids in membrane lipids of *Chlamydomonas* has been suggested to represent an adaptive reaction at low pH to decrease membrane lipid fluidity [73].

Heavy metals like cadmium, iron, copper and zinc have also been found to increase the lipid content in some microalgae [74]. The effect of high levels of cadmium was studied in *Euglena gracilis* grown as autotrophic, heterotrophic (in the dark) and mixotrophic (in the light with an organic carbon source) cultures [74]. Cadmium caused an increase in the total lipid content per cell in all three culture systems [74]. Among the membrane lipids, sterol content was lower in cadmium-treated cells cultivated under illumination. There were no changes in the total phospholipid content, although there was an increase in PG. *E. gracilis* has also been shown to display somewhat different sensitivities to copper and zinc [74]. The effect of iron on growth and lipid accumulation in *Chlorella vulgaris* was investigated by Liu *et al.* [34]. The culture in the late exponential growth phase when supplemented with Fe^{3+} at different concentrations, showed increased total lipid content of up to 56.6% biomass by dry weight [34].

Table 3**.** Examples of lipid induction in microalgae due to salinity and pH stress.

Microalgae sp	Salinity change	Lipid profile change after induction	Reference
Dunaliella salina	Transferred from 29 to 205 g/L NaCl	Increased concentration of C18 FA	[66]
Dunaliella tertiolecta	Transferred from 29 g/L to 58 g/L NaCl	Increase in lipid content and TAG	[67]
***Dunaliella* sp.**	Increased salinity from 23 to 234 g/L NaCl	Increase in total FA and monounsaturated FA	[68]
Nitzschia laevis	NaCl concentration increased from 10 to 20 g/L	Increase in unsaturated FA	[69]
***Crythecodinium. cohnii* ATCC 30556**	At 9 g/L NaCl	Increase in total FA content and DHA	[70]
Schizochytrium limacinum	Salinity at 9–36 g/L at temperature range of 16–30 °C	Saturated FA C15:0 and C17:0 was greatly increased	[71]
***Unidentified Chlamydomonas* sp.**	Low pH	Increase in saturated FAs	[73]
***Chlorella* sp.**	alkaline pH	Increase in TAG	[72]
Euglenia gracilis	Cadmium, copper, zinc	Increase in total lipids	[74]
Chlorella vulgaris	Fe^{3+}	Increase in total lipids to 56.6% of biomass	[34]

3.5. Light Irradiation Stress

Light is the most important element for photosynthesis, without which no autotrophic life can sustain or flourish. Microalgae have been reported to grow on various light intensities exhibiting remarkable changes in their gross chemical composition, pigment content and photosynthetic

activity [75] (Table 4). Moreover, different light intensities and wavelengths have been reported to change the lipid metabolism in microalgae altering the lipid profile [76] (Table 4). High light intensity leads to oxidative damage of PUFA [76], and is also required for the synthesis of C16:1 (3 *trans*) and alters the level of this fatty acid in microalgae. Typically, low light intensity induces the formation of polar lipids, particularly the membrane polar lipids associated with the chloroplast, whereas high light intensity decreases total polar lipid content with a simultaneous increase in the amount of neutral storage lipids, mainly TAGs [77–80]. High light exposure decreased the total phospholipid content and increased the level of nonpolar lipid (namely TAG) in the filamentous green alga *Cladophora* sp. [79].

In the red microalga *Tichocarpus crinitus* exposure to low light intensity resulted in increased levels of some cell membrane lipids, especially SQDG, PG and PC, whereas higher light intensities increased the level of TAG [78]. In the haptophyte *Pavlova lutheri* higher light intensities act as a catalyst to increase the lipid content and were associated with lower dilution rate-promoted increases in both cell population and weight per cell [81]. TAG production under high light conditions might serve as a protective mechanism for the cell. As outlined above, electron acceptors needed by the photosynthetic machinery might be depleted under high light conditions as well. Increased FA synthesis which in turn are stored as TAG, potentially helps the cell to re-generate its electron acceptor pool.

Light intensity not only affects the fatty acid composition in microalgae, but also the pigment composition. In the green microalga *Parietochloris incise* under low irradiance photosynthetically active radiation, cultures displayed slow growth and a relatively low carotenoid-to-chlorophyll
ratio [36]. At higher irradiances on complete medium, the alga displayed a higher growth rate and an increase in the carotenoid content, especially that of β-carotene and lutein [36].

Light/dark cycles at different growth phases also have a significant effect on algal lipid composition, as was successfully demonstrated in a detailed study on various light regimes on lipids of the diatom *Thalassiosira pseudonana* [77]. A culture grown to stationary phase under strong continuous light or under 12:12 h strong light/dark conditions had a higher amount of TAG with saturated and monounsaturated fatty acids compared to cultures grown with less light. At the exponential growth phase, however, the proportion of PUFA was highest under high light conditions [77]. This demonstrates the important role of growth phase in the accumulation of certain fatty acids. With the onset of stationary phase, algae typically show increased proportions of saturated and monounsaturated fatty acids and decreased amounts of PUFA [77]. The lipid and fatty acid compositions of three species of sea ice diatoms grown in chemostats have been analyzed and compared when cultivated at light-limiting conditions of 2 and 15 µmol photons m^{-2} s^{-1} [82]. Growing cultures at 2 µmol photons m^{-2} s^{-1} resulted in 50% more MGDG containing EPA than those grown at 15 µmol photons m^{-2} s^{-1}. Growing cultures at 2 µmol photons m^{-2} s^{-1} resulted in higher amounts of non-polar lipid bilayer-forming MGDG in relation to total bilayer-forming lipids, especially DGDG (the ratio of MGDG:DGDG increased from 3.4 to 5.7) than in cultures grown at 15 µmol photons m^{-2} s^{-1}. A shorter light period seemed to increase the production of

PUFA in *Isochrysis galbana* [83]. Sitosterol and stigmasterol were the two main sterols detected at 246.3 and 220.0 mg/100 g, respectively. A continuous increase in the level of total sterols was recorded during the life cycle at 24 h lighting [83]. The reduction of the photoperiod led to a decrease in the total sterols produced in the decay phase. A gradual increase in α-tocopherol production during the life cycle was also recorded [83]. Dark treatment caused a decrease in the relative proportion of oleate fatty acid and an increase in linoleate fatty acid in the green alga *Selenastrum capricornutum* [58]. In the dinoflagellate *Prorocentrum minimum* dark exposure led to a reduced content of TAG and galactosylglycerides, while the total content of phospholipids changed little [58].

Light irradiation can only be controlled in a closed system bioreactors or in laboratory-scale cultures, as shown by the examples above. Moreover, operational costs for controlled light add up to the production cost of biofuels from microalgae, although there are several commercial approaches of using LEDs or diverted sunlight in large-scale photobioreactors. Light is essential for TAG production, but if high light irradiation is used as a stimulant for increased TAG production, based on the examples above and in Table 4, it can be expected that this will differ for different species. In addition, TAG FA composition is different for different species in response to different light exposures. For example, in *Nannochloropsis* sp. the degree of unsaturation of FAs was lower with increasing irradiance with a significant decrease in omega-3 fatty acids (29% to 8% of total FA), caused mainly by a decrease of EPA (20:5n–3) [84]. In conclusion, light will normally stimulate fatty acid synthesis, growth and the formation of (particularly chloroplast) membranes. Therefore, the overall lipid content of algae will reflect such morphological changes.

3.6. UV Irradiance for Lipid Induction

Current research on the effect of UV irradiance in microalgae is mainly focused on the impact of UV-A and UV-B radiation on algal growth, morphology, physiology and oxidative stress [85–89], with a special emphasis on pigments and photosynthesis [90]. Examples of studies on UV radiation on lipid profiles in microalgae are shown in Table 4.

In a study carried out by Srinivas and Ochs [91] on *Nannochloropsis oculata* the effect of UV-A at different levels of exposure on total lipid accumulation was investigated. UV-A treatments significantly increased the PUFA (chlorophyll-specific lipid concentration) of *N. oculata* cells, and UV-A and decreased nutrients had a synergistic effect on lipid

accumulation. The effects of UV-B radiation on the total lipid, FA and sterol composition and content of three Antarctic marine phytoplankton species *Odontella weissflogii*, *Chaetoceros simplex* and the *haptophyte Phaeocystis antarctica* were examined in a preliminary culture experiment [92]. The cultures were exposed to constant UV-A and low or high UV-B radiation. The sterol, fatty acid and total lipid content for *Odontella weissflogii* changed little under low UV-B when compared with control conditions. In contrast, when *P. antarctica* was exposed to low UV-B irradiance, storage lipids were reduced and structural lipids increased [92]. *P. antarctica* also contained a higher proportion of polyunsaturated fatty acids under low UV-B exposure. Exposure of *P. antarctica* to high UV-B irradiance increased total lipid, TAG and FFA concentrations. Lipid concentrations per cell also increased when *C. simplex* was exposed to high UV-B irradiance [92]. This resulted from increases in FFA concentration principally saturated FA and may indicate degradation of complex lipids during high UV-B treatment [92]. Effect of UV-B radiation on lipid productivity was studied in detail in *Tetraselmis* sp. [93]. A 4 hour-exposure to UV-B radiation resulted in an overall increase in saturated FA and monounsaturated FA, whereas the PUFA content was decreased by 50% [93]. In addition, UV irradiance caused a decline in the overall rate of carbon incorporated into amino acids and a reduction in the pool size of total cellular amino acids [93]. In contrast, intracellular dissolved free amino acid increased [93].

The effect of UV radiation on growth and fatty acid composition of two diatoms, *P. tricornutum* and *Chaetoceros muelleri*, were examined in batch cultures [94]. UV radiation induced significant differences in all the major fatty acids of *P. tricornutum*. The percentages of EPA and PUFA increased while monounsaturated FA decreased in the UV-A treatment in comparison with no UV irradiance or combined UV-A+UV-B treatments [94]. On the other hand, all the major fatty acids of *C. muelleri* varied with harvest stage and UV irradiance. The percentage of monounsaturated FA in *C. muelleri* increased, while EPA and PUFA decreased under combined UV-A + UV-B treatment [94]. The study indicated that UV-A exposure may promote EPA and PUFA formation in *P. tricornutum*, whereas combined UV-A + UV-B exposure enhanced short FA and monounsaturated FA content, but suppressed PUFA formation in *C. muelleri* [94].

PUFAs, especially EPA and DHA, are abundantly synthesized by some phytoplankton species and play a key role in the marine food chain. However, they are generally considered to be sensitive to oxidation by UV radiation. *P. lutheri* and *Odontella aurita* were exposed to a combination of UV-A and UV-B radiation with a total daily dose of 110

kJ/m^2 and lipid composition was then determined on days 3, 5, and 8 of UV exposure [95]. In *P. lutheri* exposure to UV led to a decrease in the proportion of PUFAs, especially those in structural lipids (glycolipids and phospholipids) and a reduction of 20% in EPA levels and 16% in DHA levels, after 8 days; whereas for *O. aurita*, exposure to UV did not change the fatty acid composition of the total lipids and lipid fractions of the cells [95].

Interestingly, UV radiation has been suggested for microalgal lipid induction in large-scale cultivation systems. As UV radiation has genetically and physiologically deleterious effects on many life forms including microalgae [95], the impact is conceivably related to the radiation intensity. A recent study showed that the modulated use of UV-A radiation for seven days could lead to an increased production of fatty acids in *Nannocloropsis* sp. [96]. However, there is concern that constant use of UV-A light may not be viable for industrial-scale cultivation, while shorter, but stronger UV radiation could also affect microalgal lipid composition and production as a result of inhibiting nutrient uptake, carbon assimilation mechanism and damaging DNA [97].

Table 4**.** Lipid induction due to different light irradiation stress in microalgae.

Microalgae sp	Irradiation type	Lipid profile change after induction	Reference
Tichocarpus crinitus	Low light intensity	Increased TAG	[78]
Pavlova lutheri	High light intensities	Increased total lipid content	[81]
Thalassiosira pseudonana	Continuous or light/dark cycled strong light at exponential growth	Increased PUFA	[77]
Thalassiosira pseudonana	Continuous or light/dark cycled strong light at stationary phase	Increased TAG	[77]
Unidentified diatoms	Low light (2 µmol photons m^{-2} s^{-1})	50% more MGDG	[82]
Selenastrum capricornutum	Dark treatment	Increase in linoleate FA	[58]
Prorocentrum minimum	Dark treatment	Marginal increase in phospholipids	[58]
Isochrysis galbana	Shorter light period	Increase of PUFA	[83]
Nannochloropsis oculata	UV-A	Increase of PUFA, structural lipids	[91]

P. antarctica	Low UV-B	Increase in PUFA, structural lipids	[70]
C. simplex	High UV-B	Increase in total lipids	[70]
***Tetraselmis* sp.**	UV-B radiation	Increase in saturated and monounsaturated FA	[93]
Phaeodactylum tricornutum	UV radiation	Increased EPA and PUFA	[98]
Chaetoceros muelleri	UV-A	Increased monounsaturated FA	[98]
***Nannochloropsis* sp.**	UV-A	Increase in saturated FA to PUFA ratio	[96]

4. Genetic Engineering of Microalgae to Increase Lipid Production

Apart from inducing lipid biosynthesis in microalgae by external cues, some progress is emerging towards metabolic engineering towards higher TAG or omega-3 accumulation capacities. The model species *C. reinhardtii* has been the focus of most molecular, genetic and physiological research [99–106–]. Significant advances in microalgae genomics have been achieved during the last decade [103,104,106]. Expressed sequence tag (EST) databases have been established; transcriptomes as well as nuclear, mitochondrial and chloroplastidial genomes from several microalgae have been sequenced; and several more are in progress of being sequenced [102]. Of particular relevance in relation to fatty acid biosynthesis, is acetyl-CoA carboxylase (ACC) which was first isolated from the microalga *Cyclotella cryptica* in 1990 by Roessler [107] and was later successfully transformed into the diatoms *C. cryptica* and *Navicula saprophila* [108]. *ACC1* was over-expressed leading to 2-3-fold enhanced enzyme activity. These experiments demonstrated that *ACC* could be transformed efficiently into microalgae although no significant increase of lipid accumulation was observed in the transgenic diatoms [108]. It also suggests that over-expression of ACC enzyme alone might not be sufficient to significantly enhance the lipid biosynthesis pathway. For a recent review on the potential of metabolic engineering and other genetic targets to enhance lipid accumulation in microalgae, see Schuhmann *et al.* [19]. However, it should be pointed out that at present, GM strains of microalgae can only be used in small-scale closed bioreactors and are very strictly regulated. This may increase the total cost of production when compared to non-GM algae in open pond systems.

5. Conclusions and Future Directions

We have discussed the different lipid induction techniques that can be used to stimulate lipid biosynthesis, in microalgae, in particular TAG. It is clear that different microalgae species react to different stresses by producing different fatty acids or by altering their composition of fatty acids. Thus which techniques to apply for the lipid induction in particular microalgae might depend on the environmental conditions and cultivation systems. Perhaps the most obvious way to advance our understanding of how the environment can alter lipid metabolism in microalgae is to study one species under controlled laboratory conditions. Based on the literature reviewed, it is clear that amongst different lipid induction techniques, nitrogen starvation is most widely applied and studied in almost all the microalgae species that can be considered for the commercial production of biodiesel (Table 1). Change in temperature, pH, salinity and heavy metals can also induce lipids effectively, but may be difficult to regulate on a large-scale cultivation system (Tables 2–4). Rapid induction of lipid biosynthesis maybe achieved through hybrid systems where a nutrient-rich flow culture in exponential phase continuously produces algal biomass of which batches (e.g., 50%) can be transferred to low nutrient conditions but with sufficient light irradiance for photosynthesis. The lipid accumulation phase maybe further shortened by applying a combination of different induction types. For example, this may include a sudden induction, as possible in a hybrid system, combined with nutrient depletion, a salinity/pH change as well as exposure to UV irradiance. The exact combination of induction stresses that provides optimum lipid productivity in a large-scale commercial cultivation system for biodiesel production, will differ for every microalgae strain and depends on nutrient supply, environmental and climatic conditions.

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Chapter 2: Critical analysis of current microalgae dewatering techniques

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Abstract

Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. However, microalgae harvesting/dewatering is a major obstruction to industrial-scale processing of microalgae for biofuel production. The dilute nature of microalgae in cultivation creates high operational costs for harvesting, thus making microalgal fuel less economical. Within the last decade significant advances have been made to develop new technologies for dewatering or harvesting of microalgae. The choice of which harvesting technique to apply, depends on the microalgae cell size and the desired product. Microalgae dewatering process can broadly be classified as primary and secondary dewatering. This article provides an overview of current dewatering techniques along with a critical analysis of costs and efficiencies and provides recommendations towards cost-effective dewatering.

Keywords: biofuels, dewatering, harvesting, microalgae

Microalgae- Generally unicellular photosynthetic microorganisms some of which can also form a chain or colony ranging from a few micrometers (µm) to a few hundreds of micrometers.

Primary dewatering- This step aims to concentrate an algae culture from the initial biomass concentration.

Secondary dewatering (including drying) - Aims to further concentrate the slurry, increasing the total solid matter up to 90-95%.

Flocculation- Process to form algae flocs that is often performed as a pretreatment to destabilize algae cells from water and to increase the particle size before using another method such as settling or flotation.

Flotation- In this process, microalgae cells are trapped on micro-air bubbles and float at the surface of water.

Costing- For the cost analysis, harvesting of 10,000 L of *Chlorella* sp. culture was considered and was calculated by using the information from previously carried out studies and information provided by companies that supply the required chemicals and equipment to Brisbane, Australia.

Introduction

Microalgae have a robust photosynthetic capability for fixing CO_2 and converting solar energy into chemical energy. Moreover they do not need to compete with arable land and freshwater and have been considered as one of the most promising feedstocks for biofuels [1, 2]. Microalgae are typically 2-50 microns in size with a negative charge on the cell surface [3-5], but some microalgae, under certain conditions, have a larger cell size. In most cases they are motile, i.e. swimming or gliding such as dinoflagellates or raphid diatoms and form stable suspensions. Unfortunately, microalgal biomass is fairly dilute in cultures (up to 0.3-0.5 g dry biomass per litre), resulting in difficulties to harvest and dewater algae cost-effectively [6]. Microalgae harvesting can typically make up to 20–30% of the total biomass production cost [7-9]. This makes the harvesting process a major bottleneck, hindering the development of the microalgae industry. To date, there are a multitude of techniques being used for

microalgae dewatering, but with low economical feasibility. Based on their large biodiversity, microalgae harvesting processes are to a large extent species-specific [10, 11]. They are also closely linked to cell density and cultivation conditions [12].

The production of biofuel, such as biodiesel, from microalgae is a multi-step process involving cultivation, biomass harvest, lipid extraction and oil conversion. Compared to the other processes, harvesting is arguably still the most critical and challenging stage in microalgae biomass production [4, 8, 12-15]. When considering commercial-scale processes for dewatering and recovering algal biomass for further downstream processes, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting and the second is known as secondary dewatering/thickening (Fig. 1) [8-10, 16]. During the primary harvesting process, the microalgae mass ratio to water volume is increased [17]. This step aims to achieve a concentration containing 2–7% total solid matter, from the initial biomass concentration [16]. Secondary dewatering concentrates the biomass up to 15-25% which when followed by drying, aims to further concentrate the slurry, increasing the total solid matter up to 90-95%. This step is generally a more energy-intensive step than primary harvesting. Several techniques for dewatering of microalgae cultures have been developed [16]. This paper attempts to provide an overview of these techniques, to estimate their efficiencies, and then classify these techniques based on their properties. It also highlights the need for developing hybrid technology. It is desired to optimize microalgae dewatering processes by combining the strengths of several different harvesting techniques.

Primary harvesting

Primary harvesting methods reviewed here include flocculation, flotation, sedimentation, and electro-flocculation (Fig. 1) [1, 6, 18].

Flocculation

Flocculation is often performed as a pretreatment to increase the particle size before using another method (Table 1). Hence, flocculation is commonly used before secondary dewatering processes to facilitate further steps such as centrifugation or filtration [4, 10, 19]. In some cases negative charges of microalgae cells inhibit aggregation, therefore cationic

flocculants, cationic polymers and metal salts (e.g., ferric chloride, alum, aluminum sulfate, and ferric sulfate) are used to neutralize charges and facilitate aggregation [4, 13, 16, 19-22]. The efficiency of electrolytes to induce coagulation is measured by the critical coagulation concentration, or the concentration required causing rapid coagulation. Coagulation efficiency of metal ions increases with increasing ionic charge. Multivalent metal salts such as alum have been widely used to flocculate algal biomass in wastewater treatment processes [23, 24]. Alum is an effective flocculant for freshwater species such as *Scenedesmus* and *Chlorella* [25]; however, for maximizing the economic value derived from the feedstock, there is a need to produce various co-products such as pigments, protein, omega-3 fatty acids, and animal feed along with biofuel production [26]. Hence flocculation by metal salts may be unacceptable if biomass is to be used in certain aquaculture applications or to be used as food or feed. Polyferric sulphates (PFS) are reported to be a better flocculant compared to the more traditional non-polymerized metal salt flocculants as shown by Jiang *et al.* (1993) [27]. Pre-polymerized metal salts are effective over a wider pH range than non-polymerized salts [27]. Moreover, flocculation was carried out by adjustment of pH using sodium hydroxide and addition of the non-anionic polymer Magnafloc LT-25 to a final concentration of 0.5 mg L^{-1} by Knuckey *et al.* (2006) [28].

Ultrasound has also been used to induce aggregation in microalgae [29]. Microbial flocculation under nutrient depletion stress has been investigated by Lee *et al.* (2008) [30]. Flocculation happens naturally in some microalgae, for example by high light, nitrogen stress, changes in pH, salinity or the level of dissolved oxygen [22]. This typically leads to flocculation and settling and probably presents a protective survival mechanism for algae in their natural environment.

Electrolytes and synthetic polymers are typically added to coagulate (neutralize charge) and flocculate the cells, respectively [31]. Smith and Davis (2012) recently investigated auto-flocculation using magnesium-based flocculants naturally available in brackish water [32]. Moreover, magnesium-based flocculants can be obtained from wastewater treatment plants. A recent study carried out by *Taylor et al*. (2012) on *Nannochloropsis oculata* observed that by artificially treating the algae with algal extracts can not only effectively flocculate microalgae but also can increase overall lipid content [33]. Interrupting carbon dioxide to algae culture may also cause auto-flocculation [34, 35]. However, auto-flocculation may not

be as reliable as chemical flocculation [22]. Electro-coagulation flocculation (ECF) has been evaluated as a method for harvesting a freshwater (*Chlorella vulgaris*) and a marine (*Phaeodactylum tricornutum*) microalgal species by Vandamme *et al.* (2011) [36]. In this study, ECF was shown to be more efficient using an aluminium anode than using an iron anode. Moreover the efficiency of the ECF process could be substantially improved by reducing the initial pH and by increasing the turbulence in the microalgal suspension. In another study conducted by Xu *et al.* (2010) a rapid and efficient electroflocculation method integrated with dispersed-air flotation was developed for harvesting *Botryococcus braunii* with a recovery of 98.6% within 14 min [37].

When considering downstream processes to produce bio-products from algae, the use of metal salts for coagulation and flocculation poses many challenges. In wastewater sludge treatment, aluminum and sulfate have been shown to affect the specific methanogenic activity of methanogenic and acetogenic bacteria and to reduce their anaerobic digestion ability [38]. A similar problem may be faced when using algal biomass for anaerobic digestion. Land application of aluminum-treated sludge can increase heavy metal uptake and cause phosphorus deficiency in plants [39].

Natural polymers that do not raise environmental concerns may also be used as flocculants, although these are less studied. One of the most widely-used and studied natural polymers for flocculation is chitosan (at a pH around 7) that is typically derived from crab shell. Divakaran *et al.* (2002) reported successful flocculation and settling of algae by adding chitosan [40], which is considered an environmentally friendly option that has also been used in various other studies [41-43]. Other nonconventional flocculants like *Moringa oleifera* seed flour has been used by Teixeira *et al.* (2012) as another non-toxic microalgae flocculant [44]. Cationic starch is also mentioned as another potential effective flocculant for freshwater microalgae by Vandamme *et al.* (2009) [45].

Gravity-assisted sedimentation

This process is commonly used in wastewater treatment. However, this process is also appropriate for microalgae larger than 70 μm in size [16, 46], but is typically fairly slow due to the low specific gravity of algae cells [4].

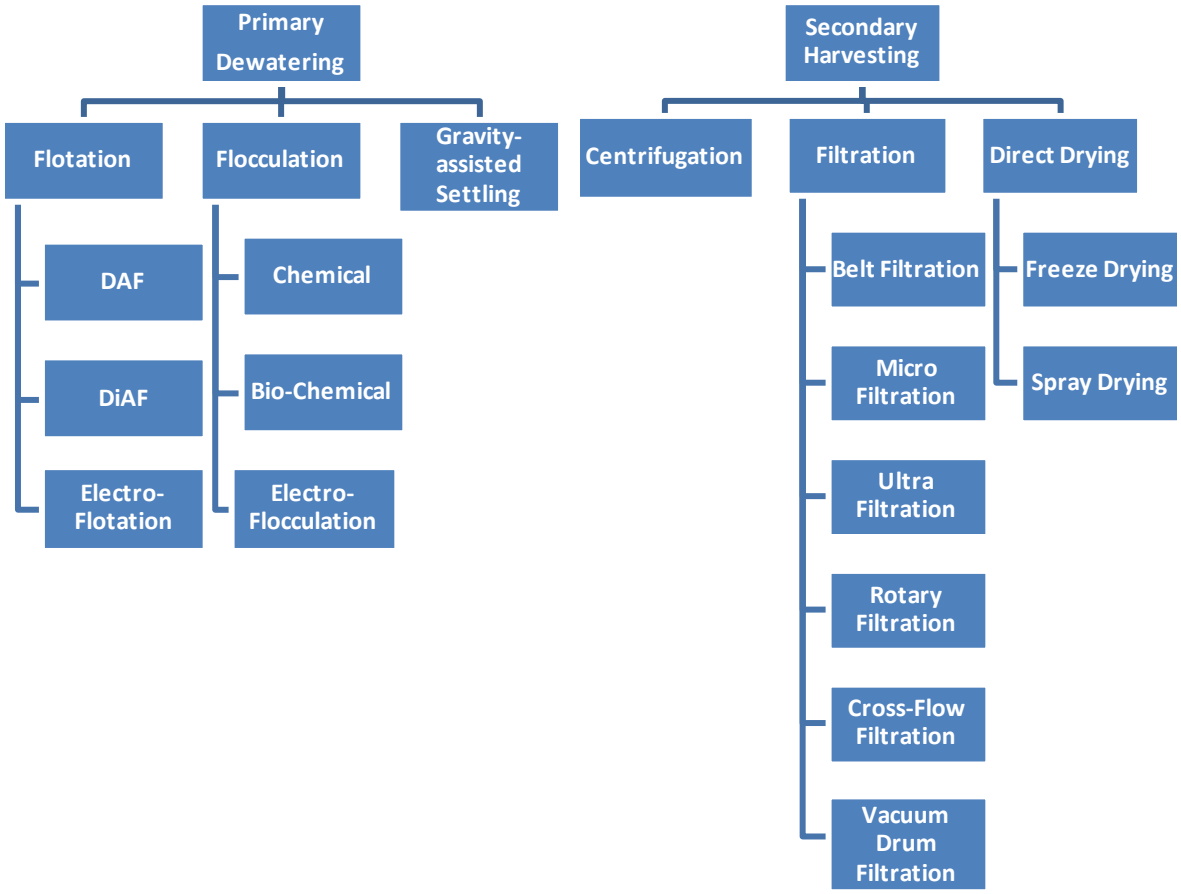

Figure 1- Overview of microalgae harvesting techniques

Table 1: Examples of various flocculation studies that have been used to harvest microalgae

ALGAE	FLOCCULANT	SOURCE	pH	DOSAGE	RESULT	REFERENCE
Tetraselmis suecica	Praestol	Acrylamide (Non-Biological)	8.2	1 mg L^{-1}	70% in 30 mins	[47]
Spirulina platensis	Praestol	Acrylamide (Non-Biological)	9.4	1 mg L^{-1}	70% in 30 mins	[47]
Rhodopseudomonas palustris	Praestol	Acrylamide (Non-Biological)	6.8	1 mg L^{-1}	86% in 30 mins	[47]
Chaetoceros calcitrans	Magnafloc ®LT25	Polyacrylamide (Non-Biological)	10.2	>1 mg L^{-1}	93% in 4 hours	[48]
Chaetoceros calcitrans	Chitosan	Inorganic polymer (Biological)	8.0	20 mg L^{-1}	83% in 4 hours	[48]
Cholrella minutissima	aluminium chloride	Inorganic salt (Non-Biological)	-	0.5 mg L^{-1}	90% in 5 hours	[49]
Scenedesmus	Greenfloc 120	Cationic starch	-	>10 mg L^{-1}	<90% in 30 + 30 mins	[45]
Phaeodactylum tricornutum	sodium hydroxide	Alkaline agent	9.8 – 10.61	-	90 – 97% in 1 hour	[50]
Phaeodactylum tricornutum	Chitosan	Inorganic polymer (Biological)	9.9	20 mg L^{-1}	90% in 30 mins	[50]
Chlorella vulgaris	magnesium + sodium hydroxide	-	10.5	0.15 mM	<90% in 30 + 30 mins	[51]

Flotation

In this process, microalgae cells are trapped on micro-air bubbles and float to the surface [16]. Efficient flotation relies on successful collision and attachment of bubbles and particles and works best when algae cells are hydrophobic [3, 52].

Dissolved air flotation (DAF) has been successfully used in water treatment plants [53, 54] and is also widely used for microalgae harvesting (Table 2). It involves the release of pressurized water (saturated with air) into the tank containing microalgae. Due to the difference of pressure, many fine bubbles form, carrying algae cells as a froth which can be skimmed off. The effectiveness of this process depends on air bubble size, solubility and the pressure difference of air, the hydraulic retention time and the floated particle size [55]. Before algae can be removed using DAF they need to be flocculated. The flocculation increases the efficiency of removal. A study carried out by Edzwald *et al.* (1993) found DAF to be more effective than sedimentation [55]. Suspended air flotation is an alternative method that could potentially harvest microalgae with a lower air:solids ratio, lower energy requirements, and higher loading rates compared to DAF [56].

In dispersed air flotation (DiAF) (or foam flotation), algae cells are floated in a mechanical cell with a high speed agitator through which a constant stream of air is passed [3]. Fine bubbles of about 1 mm diameter are generated by either “agitation combined with air injection” or “bubbling air through porous media” [57]. Hydrophobic interaction plays an important role for attachment particles, such as microalgae, to the bubbles [3]. Bubbles then rise to the surface and constantly accumulate as foam as a result of solid-liquid separation [3]. Foam fractionation is considered as an alternative to the use of expensive centrifugation for microalgae harvesting [58].

Table 2- Examples of various flotation studies that have been used to harvest microalgae.

ALGAE	SURFACTANT	SURFACTANT TYPE	pH	DOSAGE	RESULT	REFERENCE
Scenedesmus quadricauda	SDS + Chitosan	Anionic surfactant	8.0 – 5.0	20 + 10 mg L^{-1}	95% in 20 min	[59]
Chlorella sp.	CTAB*	Cationic surfactant	8.0	40 mg L^{-1}	86% in 20 min	[60]
Chlorella sp.	SDS + Chitosan	Anionic surfactant	8.0 – 5.0	20 + 10 mg L^{-1}	85-90 % in 20 min	[60]
Scenedesmus quadricauda	CTAB	Cationic surfactant	7.8	100 mg L^{-1}	>90% in 20 min	[61]
Chlorella sp.	CTAB	Cationic surfactant	9.5	1-3 mg L^{-1}	95-99 % in 11 min	[3]
Chlorella sp.	CTAB	Cationic surfactant		10 mg L^{-1}	45 min	[62]
Dunaliella salina	Aluminium sulphate Ferric sulphate Ferric Chloride	Inorganic metallic coagulants	5 5 5	150 mg L^{-1} 150 mg L^{-1} 75 mg L^{-1}	95% in 30 min 98% in 30 min 98.7% in 30 min	[63]

*Cetyl trimethyl ammonium bromide

Secondary dewatering

In secondary dewatering or thickening, the algae slurry is concentrated about 10 to 30 times and consequently the water content of the produced algae paste can be as low as 20-25 %, (Fig. 1) [13]. Energy intensive processes such as centrifugation and ultrasonic aggregation are commonly used at this stage [16]. This step requires more energy input than primary dewatering [46], and therefore needs more capital and operating costs.

Centrifugation

Centrifugation is the ideal method for rapid harvesting of algae containing high value products. Generally centrifuges can be of various types and sizes depending on the uses. A disc stack centrifuge consists of a relatively shallow cylindrical bowl containing a number (stack) of closely-spaced metal cones (discs) that rotate, it is mostly used in commercial plants for high value algal products and in algal biofuel pilot plants. Decanter centrifuges have been found to be as effective as solid bowl centrifuges for separating microalgae, but the energy consumption of decanter centrifuges is higher than that of disc bowl centrifuges at 8 kWh m^{-3} [8]. A hydrocyclone is a relatively low energy (0.3 kWh m^{-3}) particle sorting device compared to other centrifuge methods, but on the other hand it was reported to be an unreliable means of concentrating microalgae as only a maximum concentration factor of 4 could be achieved [8]. Spiral plate centrifuges are considered a relatively new generation of centrifuges, manufactured by Evodus, the suspension flows outwards in thin films over vertical plates with the solid sediment or microalgae being forced by centrifugal force to collect on the outer bottom edge of the vanes. Table 4 provides more analyses and details about the harvesting of 10,000 litres of *Chlorella* sp. with an Evodos centrifuge. More detailed studies on centrifuge harvesting have been carried out by Molina Grima *et al*. (2003) [8]. However, centrifugation is energy-intensive, not easily scalable and requires high maintenance due to fast-moving mechanical parts [8, 16, 22]. Therefore, centrifugation has high capital and operating costs and is considered too expensive for low value products such as biofuel [8, 64]. Furthermore, high speed spinning can disrupt algae cells [19, 65].

Filtration

Filtration methods such as micro-strainers, vibrating screen filters, micro-filtration and ultra-filtration have been widely studied, and have proven to be efficient (Table 3) [66-68]. One of the major disadvantages of these techniques is the high capital and operating costs to avoid filter blinding and disruptive pressure changes (high pressure or vacuum). Membrane- and ultra-filtration are costly for large-scale operations due to high operating costs for membrane replacement, clogging and pumping [4, 8, 10, 13, 16, 22, 46, 67]. Although the filtration process may be considered to be slower than centrifugation for some applications [10], it is still a simpler and lower cost alternative when compared to centrifugation, if implemented properly. Fast formation of thick filter cake which dramatically decreases flow rate, is another disadvantage of conventional filtration processes [22].

Cross-flow filtration (tangential flow filtration) has been shown to solve these problems as the filter cake is washed away during the cross-flow filtration process which increases the operation time of the filtration system [69]. However this technology is still very expensive for low value products and is not easily scalable. In addition, most studies consider the conventional filtration process as unsuitable for harvesting of small microalgae (smaller than 30 micron) [4, 10, 13, 16, 22, 70].

Drying

The water content of algal paste after secondary dewatering should not exceed 50% before oil extraction [12]. Because the cost of thermal drying is high (even higher than mechanical drying), a harvesting method with a high solid content is preferable before drying [10]. Common methods for drying microalgae after secondary dewatering are: spray-drying, drum drying, freeze-drying, and sun-drying [10]. Spray-drying is considered too expensive for low-value products such as biofuel [10]. The influence of short-term storage and spray- and freeze-drying of fresh microalgal paste on the stability of lipids and carotenoids of *Phaeodactylum tricornutum* was investigated by Ryckebosch *et al*. (2011) [76].. Solar drying is considered the most economical drying process; however it requires large land areas for large-scale operations [12, 77].

Table 3-Examples of various filtration studies that have been used to harvest microalgae

Species	Type of filtration	Effective	Reference
Coelastrum sp.	Non-precoated vacuum drum filter	18% TSS	[6]
Coelastrum sp., *Scenedesmus* sp.	Potato starch vacuum drum filter	37% TSS	[6]
Coelastrum sp.	Belt filter	9.5% TSS	[6]
Chlorella and *Cyclotella*	Micro filtration	-	[55]
Scenedesmus quadricauda	Ultra-filtration membranes	-	[71]
Spirulina sp.	Ultra-filtration membranes	20%TSS	[72]
Spirulina micractinium	Rotary vacuum filter	1-2% TSS	[73]
Spirulina	Belt filters	18 % TSS	[8, 74]
Haslea ostrearia, Skeletonema costatum	Cross-flow microfiltration and ultra-filtration	-	[75]

Techno-economic assessment

Using the information from previously carried out studies and specifications provided by companies that supply the equipment and chemicals, a theoretical calculation was carried out to determine the techno-economic feasibility of overall biomass recovery in a one-step as well as a two-steps method. For the costing purpose, harvesting of 10,000 L of *Chlorella* sp. culture was considered (Table 4). Assessments were independently developed in accordance with Australian conditions and where possible, were compared to equivalent costing from previous economic analyses of microalgae biofuel systems.

Table 4 - Cost of harvesting 10,000 L of *Chlorella* sp. with different harvesting techniques

	Single step		**Primary**				**Secondary**	
	Centrifugation		**Sedimentation**	**Flotation (1)**	**Flotation (2)**		**Filtration**	**Centrifugation**
Total Energy Consumed	55 kWh/10 $m^{3^{\wedge}}$		**-**	7.4-8.4 kWh/10 m^{3+}	0.150 kWh/10 $m^{3!}$		1 – 3 kWh/m^3 [4]	5.5 kWh/$m^{3^{\wedge}}$
Energy Cost (AUD)[$]	$12.10*		**-**	$1.62 – $1.84*	$0.033		$0.22 – $0.66*	$1.21*
Dosage required	**-**		100 g @ 10 mg L^{-1} [1]	30 g @ 3 mg L^{-1} [3]	100 g @ 10 mg L^{-1}		**-**	**-**
Chemical Cost (AUD)	**-**		$2.50 (Chitosan @ $25/kg)	$0.24 (CTAB @ $8/kg)	$0.8 (CTAB @ $8/kg)		**-**	**-**
pH adjustment Dosage	**-**		1.5 to 2 L acetic acid~	**-**	-		**-**	**-**
pH Adjustment Cost	**-**		$1.20 – $1.60 @ $800/ ton	**-**	-		**-**	**-**
Total Cost (AUD)	**$12.10**		**$3.70 - 4.10**	**$1.86 – $2.08**	**$0.833**		**$0.22 – $0.66**	**$1.21**

[$] Australian Dollar (= approx. US $1.04)
[*]Electricity prices were calculated based on $0.22 per kWh
[^] An Evodos centrifuge was used for this study
[+] Flotation cell considered is Jameson cell and energy consumption was determined using various published studies and our own published [3} and unpublished data.
[!] Flotation cell considered is Column flotation cell and energy consumption was determined using work done by Coward *et al*. (2013) [62]
[~] The volume was estimated by doing an experiment with 1 L of algae culture and mathematical calculation.

The above table compares some of the traditionally used harvesting methods in microalgae bioprocessing. From the table it can be summarized that due to its high energy consumption, single step centrifugation is the most expensive method when compared to other techniques.

Flotation appears to be the most cost-effective method for primary dewatering; however if used with centrifugation the overall setup costs will increase and would result in higher capital costs. On the other hand for flotation, if used in conjunction with filtration, the overall process may become more feasible, but there is still room for improvement. Moreover, the CTAB chemical used for flotation is not only toxic to the environment but also makes the biomass unfit for human and animal consumption. Flocculation coupled with filtration may be more cost-effective, but chitosan used for flocculation is bio-degradable, as it is derived from a biological source (crustacean). However, large-scale use of chitosan may not be possible as it is expensive as well as this would put pressure on crustacean populations. Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including *Pandalus borealis*. Hence some harvesting techniques are more feasible than others, when considering costs only, but some of these may not be environmentally friendly. Thus there is a need to optimize current methods or to develop improved methods which are not only cost-effective but also environmentally friendly.

Classification of current harvesting processes

Current harvesting methods mentioned above can be divided into chemically-based, mechanically-based and biologically-based categories (Fig. 2). Various combinations or sequences of these methods can be used for cost-effective harvesting. Currently biologically-based methods are being investigated as a cost-reducing and environmentally friendly means of harvesting [78]. In any case, it needs to be checked if any desirable valuable compounds are lost during the process. To develop a cost-effective harvesting technique, apart from the costs, one has to consider the following three main aspects: (1) species-specific requirements of microalgae that need to be harvested, (2) recovery/yield of desired product and (3) environmental impact.

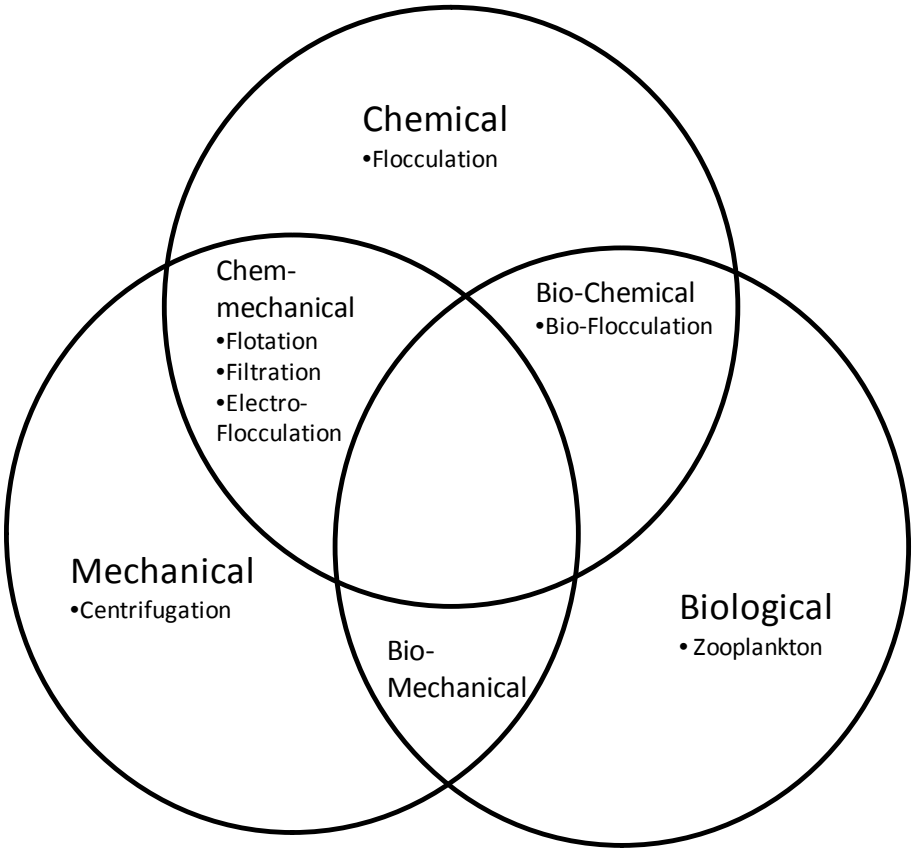

Figure 2- Interaction between different harvesting techniques.

Chemically-based method can be termed as a harvesting method that involves addition of chemicals to the microalgae culture to induce flocculation, which is used in various solid–liquid separation processes as a pre-treatment stage [79]. The chemical reactions are highly sensitive to pH, and high doses of flocculants are required to produce large amounts of sludge which may leave a residue in the treated effluent. Though being cost-effective a major disadvantage could be the presence of harmful salts and chemicals in the extracted biomass which can possibly pose health and environmental risks. For example use of aluminium oxide to flocculate microalgae can lead to accumulation of aluminium salt precipitates in the biomass.

Mechanical harvesting as the name suggests is the method that involves the use of a mechanical machine to harvest microalgae which generally includes centrifugation, filtration and flotation. Molina Grima *et al.* (2003) [8] concluded that centrifugation is a preferred method for harvesting of microalgal biomass, especially for producing extended shelf-life concentrates for aquaculture, pharmaceuticals and other high value products like omega-3. However, Knuckey *et al.* (2006) state that exposure of microalgae cells to high gravitational and shear forces can damage the cell structure [28]. In addition, processing a large amount of culture using centrifugation is time-consuming and increases the overall

costs of microalgae biomass production (Table 4). Filtration and gravitational sedimentation are widely applied in wastewater treatment facilities to harvest relatively large (>70 μm) microalgae such as *Coelastrum* and *Spirulina.* However, they cannot be used to harvest algae species approaching bacterial dimensions (<30 μm) like *Scenedesmus, Dunaliella* and *Chlorella* which can rapidly and easily blind the filter [16]. This may result in higher operating costs and frequent replacement of filters. In summary, most technologies including chemical and mechanical methods, greatly increase operational costs for algal production and are only economically feasible for production of high-value products [80].

Biological harvesting is the method in which bioproducts or other microorganisms are used for harvesting of microalgae. When cultivating microalgae, some cultures tend to aggregate and grow as fluffy pellets, tightly packed, compact or dense granules. These fluffy pellets are caused by filamentous microorganisms, including some species of molds and bacteria [81-83], and may assist in trapping additional microalgal cells, one of the major advantages of cell pelletization [7, 78, 83]. Fungal cell growth can be induced by changing operational conditions during cell cultivation, rather than using $CaCO_3$ powder or other nuclei to induce the fungal pelletization [81] which are costly and cause solid waste disposal issues. A preliminary study was recently conducted by Zhou *et al.* (2012) [83] to inoculate filamentous fungal spores when culturing mixotrophic green algae, *Chlorella vulgaris*, with the result that pellets clearly formed within two days of culture. Microalgae cells, aggregated together with fungal cells, were immobilized in the pellets [82]. Bio-flocculation using flocculating microalgae has also been investigated by others [78, 83]. The advantage of this method is that neither addition of chemical flocculants is required nor the cultivation conditions have to be changed. This method is as simple and effective as chemical flocculation; however it is potentially more sustainable and cost-effective. No additional costs are involved for pre-treatment of the biomass before oil extraction and for the medium before it can be re-used [27]. An interesting method is the use of zooplankton to harvest microalgae [84]. Biological harvesting could be a cost-effective method to harvest microalgae, but it is time-consuming and has limitations in large-scale cultivation as enough bio-product must be co-produced. Also chances of cross-contamination are very high.

Based upon the current study conducted on microalgae harvesting technologies, it is evident that harvesting techniques should not only be cost-effective and rapid, but also

have to be environmentally safe and easily scalable for a microalgae-based bio-refinery industry. Thus there is a need to think outside the box and develop new hybrid methods that may combine the best aspects of several techniques (Fig. 2).

Bio-chemically-based methods

As described above flocculation assisted by chitosan (biologically-derived) has been used in many studies on different microalgae and has proven to be very promising [41, 85, 86]. Another example of biologically-derived flocculation is the use of *Moringa oleifera* seeds, which have also been used for water treatment due to their high flocculation potential, low cost and low toxicity. Recently, Teixeira *et al.* (2012) demonstrated *M. oleifera* as a successful flocculating agent for *Chlorella vulgaris* [44]. In addition, a range of new bioflocculants are proposed to address the cost and environmental concerns for current flocculation methods [14]. Microalgae flocculation was also achieved by using naturally-available ions in brackish water, and a variety of precipitating ions, including Mg^{2+}, Ca^{2+} and CO_3^{2-} can lead to auto-flocculation of microalgae [32]. A combination of bioflocculants together with a low dose of chemicals may lead to the best flocculation outcome.

Emerging technologies

When considering chemical, mechanical and biological harvesting methods, each method has its advantages and disadvantages. Bio-mechanical and chemical-mechanical methods for flocculation are less explored when compared to other methods. Developing hybrid techniques which make use of all three harvesting categories may be a viable option that is worth exploring.

The conceptual photobioreactor shown by Chen *et al.* (2011) has the potential to be developed into a commercially viable microalgae cultivation system with zero electricity consumption [66]. This was made possible by combining sunlight and multi-LED light sources with solar panels and a wind power generator. Similarly when considering harvesting, electricity cost is the key factor that makes the process costly, but renewable energy sources such as solar and wind can be used to generate green electricity [87]. The main disadvantage of these systems is the high construction costs.

Another option to reduce the cost of harvesting could be by combining two or more stages of microalgal biodiesel production with a harvesting method into one step, for example the

study carried out by *Taylor et al.* (2012) [33]. By doing so, not only the cost can be reduced but also the overall time required for a full production cycle can be reduced. For example, developing a process that can help in rapid induction of lipids as well as flocculation could accelerate the harvesting process. Similarly, a method was developed by Hejazi *et al.* (2004) for milking β-carotene from *Dunaliella salina* in a two-phase bioreactor [88]. In this technique, cells were first grown under normal growth conditions and then stressed by excess light to produce larger amounts of β-carotene and later a biocompatible organic phase was added and the β-carotene was extracted selectively via continuous re-circulation of a biocompatible organic solvent through the aqueous phase containing the cells. Because the cells continue to produce β-carotene, the extracted product was continuously replaced by newly produced molecules. Therefore, the cells are continuously reused and do not need to be grown again. Thus, in contrast to existing commercial processes, this method does not require harvesting, concentrating and disruption of cells for extraction of the desired product [88, 89].

Matrix-attached algae culture systems have been developed for growing microalgae on the surface of polystyrene foam to simplify the cell harvest [7, 90]. These methods are innovative and will decrease the harvesting costs to some extent if developed successfully, but require heavy investments on equipment and chemical supplies with various combinations or sequences of these methods. Xu *et al*. (2011) developed a simple and rapid *in situ* magnetic harvesting method by using Fe_3O_4 nanoparticles on *Botryococcus braunii* and *Chlorella ellipsoidea*. Magnetic particles were added to the microalgal culture broth and then separated by an external magnetic field [91]. Recently a GM approach has also been used for harvesting microalgae of genera *Chlamydomonas*, *Dunaliella*, *Scenedesmus* and *Hematococcus* sp. [91].

Conclusion

When considering the research carried out in the field of harvesting microalgae over the past few decades, much progress has been made. Researchers have optimized various techniques; machines have become more energy-efficient. There is a need to optimize current methods or to develop improved techniques which are not only cost-effective but also environmentally friendly. Moreover, there is a need to develop hybrid harvesting technology that can use the best of all current harvesting methods. The costing calculation in this review suggests that flotation for primary dewatering coupled to filtration maybe the

most cost-effective method for microalgae harvesting, but this maybe different for different microalgae strains. The comparison also highlights the fact that none of the harvesting methods is cost-effective when considering cultivation of microalgae solely for biodiesel production. Hence it is a necessity to derive a secondary product which has a higher market value when compared to biodiesel. In the past, the majority of studies have focused on freshwater microalgae species and not much work has been done on marine species. With limited availability of freshwater, further research should be focused more on the processing of marine microalgae.

Future perspective

Rapid depletion of fossil fuels and rising greenhouse gas emissions have made the case of microalgae as a biofuel source even more compelling. Moreover, microalgae grown on non-arable land have great potential for provision of animal feed, and microalgae can also be used for wastewater purification. At present, harvesting technologies are costly and labour-intensive but recent studies indicate that major efforts are underway to develop new, more efficient and cheaper harvesting technologies, many of which will be microalgae strain-specific. Microalgae are being grown in outdoor ponds, greenhouses, photo-bioreactors, fermenters and hybrid systems combining bioreactors and ponds. As more and larger microalgae pilot plants will be in operation within 5-10 years, more accurate economic assessments of different harvesting methods will be possible that will feed into the life cycle analyses of future algal biorefineries. With the availability of new and more efficient harvesting systems, microalgae harvesting will be less costly, easier to manage and more accessible for farmers, rural communities and industry around the world. Microalgal biorefineries are expected to be first established at large-scale in countries with (1) high irradiation, (2) flat, non-arable, desert, saline or low biodiversity land and (3) access to water unsuitable for human consumption or irrigation (brackish, marine or polluted).

Executive summary

Background

- Microalgae harvesting/dewatering is a major obstruction to industrial-scale processing of microalgae for biofuel production.

- When considering commercial-scale processes in order to dewater and prepare algal biomass for further downstream processing, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting and second is known as secondary dewatering/thickening.

Classification of harvesting techniques

- Primary harvesting includes flocculation, flotation and sedimentation. Whereas, secondary harvesting includes centrifugation and filtration.
- Current harvesting methods can be classified into chemically-based, mechanically-based and biologically-based categories.

Costing and analyses of current harvesting techniques

- Single step centrifugation is the most expensive method when compared to other techniques.
- Flocculation coupled with filtration may be cost-effective, but chemicals used in flocculation of microalgae may lead to environmental damage.
- Flotation could be the most cost-effective method for primary dewatering; however if used with centrifugation the overall setup costs increase and result in higher capital costs.

Future perspective

- There is a need to think outside the box and develop new hybrid methods that use best-of-all techniques and are not only cost-effective, but also have a low environmental impact.

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Chapter 3 - Rapid lipid induction in *Chlorella* sp. by UV-C radiation

Overview

Previous UV radiation research in microalgae is mainly focused on the impact of UV-A and UV-B radiation on algal growth, morphological and physiological changes and triggered oxidative stress. Compared to UV-A and UV-B, UV-C is more energetic and it was anticipated that UV-C radiation could be more efficient for lipid stimulation in microalgae. In this chapter, we used *Chlorella* sp. BR2 exposed to instant UV-C radiation as a module to study the efficiency of UV-C radiation on algal lipid stimulation.

Key Findings

- Short dosage of UV-C radiation (100 and 250 mJ/cm^2) can be used to induce lipids in *Chlorella* sp. BR2 within 24 h.
- Nearly double the amount of total lipids were present in the culture radiated at 100 mJ/cm^2 and 250 mJ/cm^2 with significant increases in total unsaturated fatty acids.
- UV-C lipid induction technique could be used to induce polyunsaturated fatty acids (PUFA's) biosynthesis.

Chapter 3 - Rapid lipid induction in *Chlorella* sp. by UV-C radiation (Submitted)

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Abstract

In this study we present a novel approach to induce lipids in *Chlorella* sp. within 24 h by short term UV-C radiation (UVR) stress at different energy intensities ranging from 0-1000 mJ/cm^2. Increase in the lipid fluorescence was measured by Nile red staining and fluorescence-activated cell sorting analysis followed by gas chromatography-mass spectrometry. Lipid fluorescence was significantly increased in cultures radiated at or above 250 mJ/cm^2 compared to the mock-treated control cultures. Lower dosages at 100 mJ/cm^2 and 250 mJ/cm^2 led to a near doubling of total fatty acids, with a significant increase in total unsaturated fatty acids. This study provides a protocol for rapid lipid induction of microalgal cells by UV-C and the possible impact of UV-C radiation on fatty acid metabolism.

Keywords

Chlorella, Lipids, Microalgae, PUFA, UV-C

Introduction

Microalgae have become increasingly important feedstocks for different industrial processing applications like biofuel, high valve compounds and carbon sequestration [1,2]. The interest in microalgae for these potentials predominantly focuses on their high growth rates [3] and high lipid content, especially non-polar triacylglycerides (TAG) suitable for biodiesel production [4]. Being secondary metabolites, however, the production of TAGs is normally not compatible with the high growth rates in microalgae. Therefore, finding suitable methods to increase the lipid production without challenging algal growth rates is one of the major obstacles hindering the development of microalgae industrial application.

As microalgae have immense diversity, screening for optimal strains is the most important criteria for their potential utilization, particularly for uses in valuable oil or biofuel applications strains should display relatively high lipid contents, fast growth and easy

cultivation and harvesting properties. *Chlorella* sp. is successfully cultivated at large-scale for commercial purposes and is considered a good feedstock for algal biodiesel production [5]. Hence, there are many attempts to increase lipid production/productivity in these species. For example, lipid content of *C. vulgaris* could be significantly increased by 40% in low nitrogen medium [6]. With manipulated culture conditions of 1.0 mM KNO_3, 1.0% CO_2, 60 µmol photon $m^{-2}s^{-1}$ and 25^{o}C, the lipid production of *C. vulgaris* was further increased 2.5-fold [7]. In addition, the lipid stimulation in *Chlorella* sp. was also achieved via silicon deficiency [8] and iron supplementation [9]. Similar to these autotrophic culture conditions, the yield of bio-oil from heterotrophically-grown *C. protothecoides* increased 3.4 times and made up to 58% of dry weight [10-12]. When co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*, about 4-fold lipid production was obtained in *C. vulgaris* [13]. Although these protocols highlight the possibility to increase lipid production based on laboratory research, there are still many concerns for their promotion and application, such as the difficulty of maintenance, elongated cultivation periods leading to low lipid productivity, high cost for large-scale cultivation and potential environmental impacts. In most cases, it is also uncertain whether the induced lipid production during cultivation was derived from sacrificing of algal biomass growth or not. Therefore, there is a need to develop an effective technique to rapidly stimulate lipid production, while sustaining high cell growth.

Different from the conventional cultivation protocols that aim at biomass, accumulation of lipids in microalgae has been achieved mainly under unfavorable and stressful conditions [14-16]. Due to the disturbance on normal metabolism of membrane phospholipids during rapid cell division, stress can induce cessation of cell division and diversion of photosynthetic energy into TAG production [15]. In addition to nutrient starvations, stresses also include temperature and pH changes as well as high irradiance (e.g. UV-light) [17,15]. In comparison, UV radiation (UVR) is a sound protocol for microalgae lipid induction in large-scale cultivation. As UV radiation has genetic and physiological deleterious effects on many life forms including microalgae [18], the impact is conceivably related to the radiation intensity. A study conducted by Forján et al. showed that the modulated use of UV-A radiation for seven days could lead to increase in production of fatty acids in *Nannochloropsis* sp. [19].

Previous UVR research in microalgae was mainly focused on the impact of UV-A and UV-B radiations on algal growth, morphological and physiological changes and triggered oxidative stress [19-25]. Compared to UV-A and UV-B, UV-C has more energetic radiation

which is believed to have more adverse impacts on microalgae. UV-C has recently been applied to co-stimulate microalgal lipid production in *Tetraselmis* sp. together with nutrient stress at the end of its growth phase, resulting in an increase in long chain-polyunsaturated fatty acids (LC-PUFA), especially C18:4, including a two-fold increase of ω-3 fatty acids [26]. Therefore, to find a rapid lipid induction system for microalgae, we investigated application of UV-C stress to microalgae whenever the biomass/growth is optimal during nutrient replete conditions, rather than at the end of the growth phase.

In the current study, we used *Chlorella* sp. exposed to instant UV-C radiation as a module to study the efficiency of UV-C radiation on algal lipid stimulation. The variation of lipid production was quantified by fluorescence intensity changes in the cells by Nile red staining. Changes in individual fatty acid composition were further quantified by GC-MS analysis, demonstrating a clear and rapid significant induction at a dose of 250 mJ/cm^2 UV-C. In the meantime, the deleterious impact on algal cells was also measured by cell survival rate and chlorophyll pigments quantification.

Materials and methods

Microalgae cultivation and UV-C treatment

Chlorella sp. BR2 culture was originally collected from the Brisbane River (GPS coordinates South -27° 31' 21.36" East +153° 0' 32.87") and was cultured in the Algae Biotechnology Laboratory at The University of Queensland as previously described [27]. Primary stock cultures were maintained aerobically in 100 ml Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent white light (120 µmol photons m−2s−1). For experiments, *Chlorella* sp. BR2 was grown in f/2 medium [28] in autoclaved tap water. The culturing conditions were set at 23°C on an orbital shaker (Thermoline) at 100 rpm with 12 hours of light and dark rhythm. When the cell density reached 1.6x10^7/mL, which was still nutrients replete and in the late exponential growth phase for this strain [26], the culture was used for UV-C radiation trials. After gently stirring, 5 mL aliquots of *Chlorella* sp. BR2 culture were pipetted into a Petri dish, forming a thin layer inside (total of 20 plates). Plates were randomly divided into five groups with three plates used for each for UV-C radiation (253 nm) treatment in a UV chamber, with dimension of 31.7 x 24.1 x 15.2 cm containing five G8T5 format, minibipin bulbs (Biorad, Gs-Genelinker, California, USA). They were separately irradiated at 0, 100, 250, 500 and 1000 mJ/cm^2 (1 J/s= 1 Watt) as described previously [26 Algae survival rates were measured by counting the live cells based on visibly intact chloroplasts in each

replicate. The cell size was also measured by compound microscopy (Olympus). One of four Petri dish cultures was randomly selected and used for thin layer chromatography (TLC) analysis as suggested by Timmins et al, [29]. Briefly, all cells were centrifuged at 4000xg for 5 min and dried at 70°C overnight. The dried algal cells were grinded and dissolved in chloroform, methanol and water (v/v/v; 1:2:0.8). The extraction was filtered (0.22 um) and chloroform and water (v/v; 2:1.8) were added for phase separation. Then, the lipid-containing supernatant layer was transferred into an Eppendorff tube and the chloroform was evaporated in a desiccator. While 20 µL chloroform were added to resuspend the extracted lipid, 3 µL were loaded on a 10 x 10 cm HPLC-HL silica gel plate (Anatech) (n=3). The mobile phase consisted of n-hexane, diethyl ether and acetic acid (v/v/v; 80:20:2).

Lipid fluorescence analysis and GC-MS analysis

For each replicate, 1 mL of algal cells was sampled and stained with 3 µL Nile Red solution (a 10 mg/mL Nile Red (Sigma, USA; stock solution was prepared in acetone and stored in the dark at 4°C). After 20 min incubation in the dark, the lipid fluorescence intensity of cells was detected by fluorescence-activated cell analyzing (BD LSR II: Analyzing flow cytometer) with 573 nm of excitation wavelength. A total of 10,000 cells were counted in each sample. A gate was set up to separate the fluorescence-activated cells and inactivated cells based on the analysis of cells without Nile Red staining. The background absorbance from chloroplast auto-fluorescence was subtracted from each sample using mock-treated control cultures.

GC-MS analysis was carried using 4 mL of algal culture per sample (n=3). Each plate was centrifuged at 16,000xg for 3 min. The supernatant was discarded and the lipid in the algal pellet was hydrolysed and methyl-esterified with a 300 µL of 2% H_2SO_4 methanol solution at 80°C by shaking (480 rpm) for 2 h on a thermalmixer (Eppendorf). Prior to esterification, 50 µg of heneicosanoic acid (C21) were added to the pellet of each sample as an internal standard. After esterification, 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane (analytical grade) were added and vortexed for 20 s. Phase separation was achieved by centrifugation at 16,000xg for 3 min and the hexane layer was used for carotenoid profile analysis. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out on an Agilent 6890 GC coupled to a 5975 MSD. A DB-Wax column (Agilent, 122-7032) was used with running conditions as described in Agilent's RTL DBWax method (Application note: 5988-5871EN). Identification of fatty acid methyl esters (FAME) was based on mass

spectral profiles and retention times in the Agilent's RTL DBWax method. Each FAME was quantified using the formula:

Fatty acid (µg/mL) = (Total ionic current of fatty acid/ Total ionic current of standard) x (molecular mass of fatty acid/molecular mass of internal standard) x 50/4

Microscopic analyses

After the UV-C carotenoid induction phase, microalgal cells were stained with 2 µg/mL Nile Red (dissolved in acetone; Sigma, USA) for 15 min and photographed using a fluorescent Olympus BX61 microscope fitted with a 100 W High Pressure Mercury Burner and an Olympus DP10 digital camera. Differential interference contrast and epifluorescent (excitation: 510–550 nm, emission: 590 nm) images were obtained at 40× magnification.

Analytical methods

Data for lipid productivity was statistically analysed by one-way analysis of variance (ANOVA) using lipid productivity as dependent variables and different microalgal cultures as the source of variance. This was succeeded by Turkey's multiple comparisons test (P<0.05) where appropriate.

Results

UV-C–irradiation stimulates microalgal lipid accumulation but increases cell mortality

To test whether rapid lipid induction could be achieved on actively dividing cells, *Chlorella* sp. BR2 cultures during exponential growth phase were treated with various doses of UV-C radiation. As expected for rapidly dividing cells, lipid yellow fluorescence after Nile red staining was rarely observed among the cells in the control cultures while lipid fluorescence intensities were increasingly stronger with higher of UV-C radiation doses (Fig. 1a). At higher doses, cell rupture was also observed starting from 500 mJ/cm^2 and became more prominent at 1000 mJ/cm^2, as shown by the presence of free small yellow lipid bodies released into the medium (Fig. 1a). Results obtained from TLC also confirmed the Nile red observations, where a bright band of TAGs was visible for cultures treated at 100 and 250 mJ/cm^2 (Online Resource Fig. 1). As expected the algal survival rate decreased gradually with increasing UV-C radiation (Fig. 1b). Cell survival rates reached half at a UV-C radiation of 100 mJ/cm^2 (LD50), and at 500 mJ/cm^2 to 1000 mJ/cm^2 only 5% of the cells had survived, respectively. By contrast, the cell size gradually became larger with an increase of UV-C radiation (P<0.05, Fig. 1c).

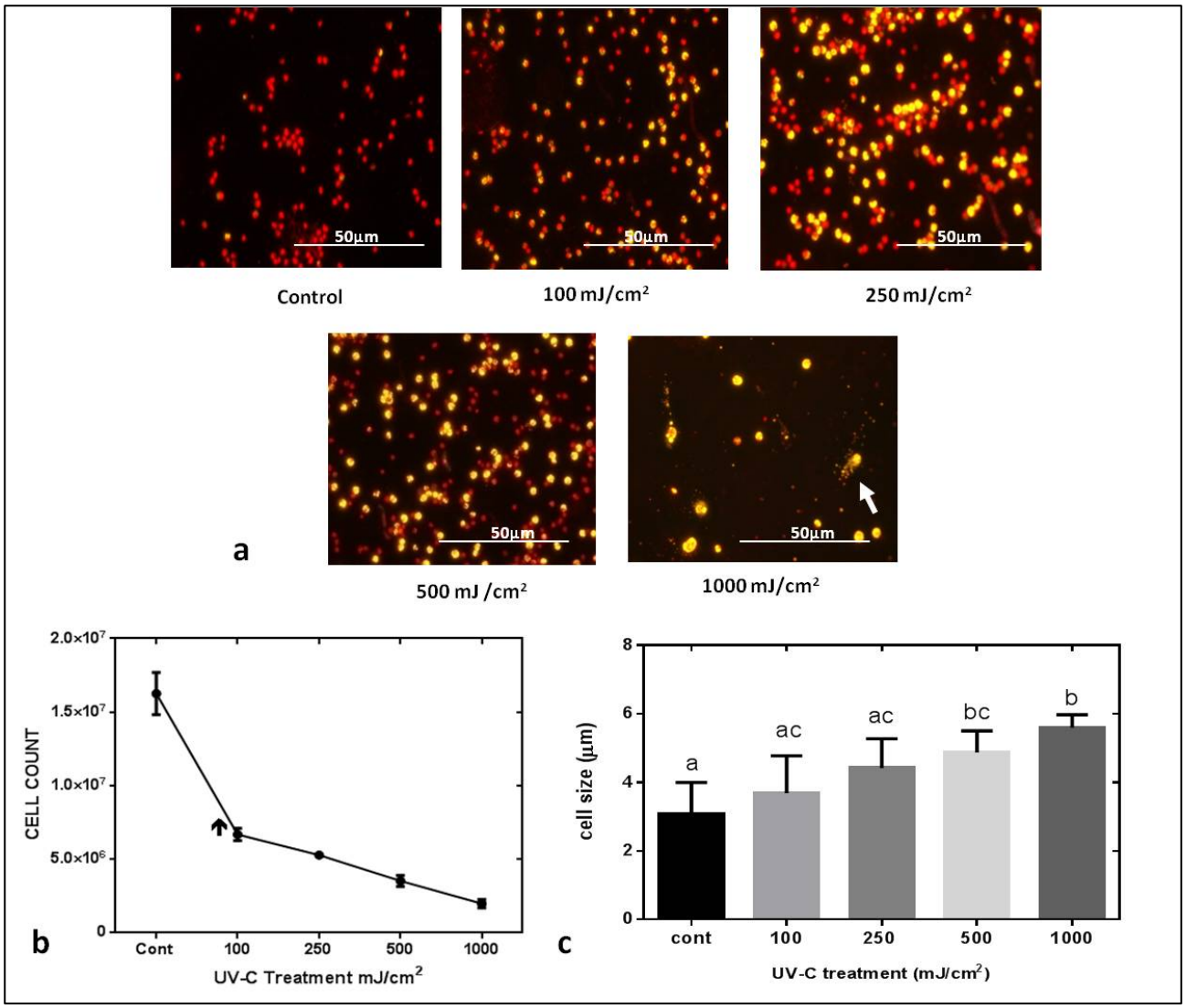


Figure 1: a) Nile red-stained *Chlorella* sp. BR2 cells observed at 40x magnification with a scale size of 50 µm. Cells with maximum lipid fluorescence (shown as yellow) were observed at 250 and 500 mJ/cm^2 UV-C radiation. As the radiation was increased to 1000 mJ/cm^2 cell rupturing was apparent (white arrow) with lipid bodies released into the medium. b) Killing curve of *Chlorella* sp. BR2, showing the number of cells that survived treatment with UV-C at certain UV-C radiation doses. Values are mean ± standard error (n = 3). c) Cell size of *Chlorella* sp. BR2 after different UV-C treatments. Values are mean ± SE from three separately grown cultures (n = 3). Different letters indicate significance differences ($P < 0.05$).

UV-C induces lipid fluorescence in *Chlorella* populations

To profile and quantify lipid accumulation in *Chlorella* cells following different UV-C exposure, a flow cytometer was used (Online Resource Fig. 2). With an increase of UV-C radiation, algal cells were divided into two different populations, named P1 and P2, according to a background fluorescence intensity cutoff in the unstained control population. The majority of the P1 population in this control was attributed to auto-

fluorescence of chloroplasts (Fig. 2a). In the untreated, but Nile red-stained control and in the culture treated with 100 mJ/cm^2 UV-C, 99% of the cells were attributed to the P1 population, whereas only 1% were present in the P2 population (Fig. 2a,b). When UV-C radiation of 250 mJ/cm^2 was used for treatment, P1 was reduced to 25%, while the lipid-accumulating P2 increased to 74%, indicating a marked increase in lipid production after UV-C treatment (Fig. 2a). However, at 500 and 1000 mJ/cm^2 only 3% of the cells were present in the P1 population and nearly all (97%) cells were present in the P2 population. The P2 population started to split into another two clusters at UV-C radiations of 250 mJ/cm^2 and 500 mJ/cm^2 (Online Resource Fig. 2). However, this tendency was not apparent at 1000 mJ/cm^2 where only one cell cluster was observed.

Quantification based on average fluorescence intensities from three separately-grown and -treated microalgal populations, showed that P1 cells always had much lower fluorescence intensities than the lipid-accumulating P2 populations (Fig. 2b). Compared to the mock-treated control, the lipid fluorescence intensity of algal cell was significantly increased with an increase in UV-C radiation. However, there was no significant difference between the control and the 100 mJ/cm^2 treatment (Fig. 2b,c). When UV-C radiation was applied at 250 and 500 mJ/cm^2, the total lipid fluorescence nearly increased by 30 and 70 times respectively. Subsequently, the lipid intensity of P2 cell sharply dropped to the control level at 1000 mJ/cm^2 (Fig. 2c). However, the total fluorescence was increased nearly 20 times at 1000 mJ/cm^2 when compared to control. It should be mentioned that this strong increase in lipid fluorescence was probably influenced by the presence of free lipid bodies at higher UV-C dosages (Fig. 1a) and not a direct measure of cellular lipid accumulation.

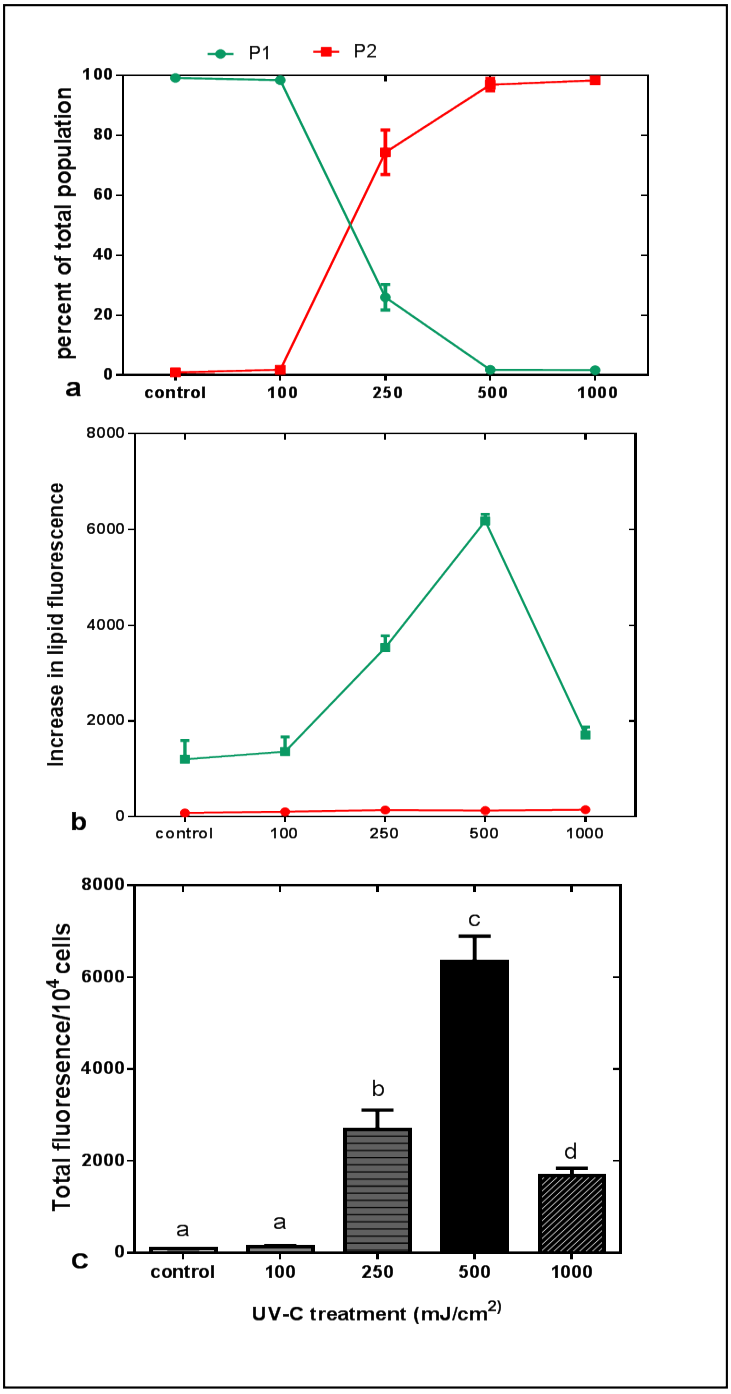


Figure 2: FACS analysis of *Chlorella* sp. BR2 at 24 h after receiving different UV-C dosages. (a) Cells present in P1 and P2 population (b) Lipid fluorescence of P1 and P2 population (c) Total fluorescence of 10,000 cells at different UV-C dosage. Values are mean ± SE from three separately-grown cultures (n = 3). Bars with different letters indicate significant differences (P<0.05).

UV-C treatment induces production of polyunsaturated fatty acids (PUFA)

To further quantify fatty acid contents and profile individual fatty acids, GC-MS was carried out on algal cultures. The results obtained from GC-MS analyses confirmed the results obtained by flow cytometry, showing a significant increase in total fatty acid in UV-C-treated cultures (100 and 250 mJ/cm^2) compared to untreated controls, whereas cultures treated with 500 and 1000 mJ/cm^2 showed no significant difference (Fig. 3). Amongst the saturated fatty acids (SFA), the highest increase was observed for C16 (palmitic acid), followed by C20 and C14 (Online Resource Fig. 3). On the other hand, C12 (lauric acid) did not show any increase in cultures treated with 100 and 250 mJ/cm^2 UV-C, whereas in cultures treated with 500 mJ/cm^2 the amount of C18 was significantly higher when compared to the mock-treated control cultures. When comparing different unsaturated fatty acids (USFA), cultures treated with 100 and 250 mJ/cm^2 UV-C showed significant increases for all detected USFA, most notably C16:1, C16:2, C18:1 , C18:3 and C20:4 (Online Resource Fig. 4).

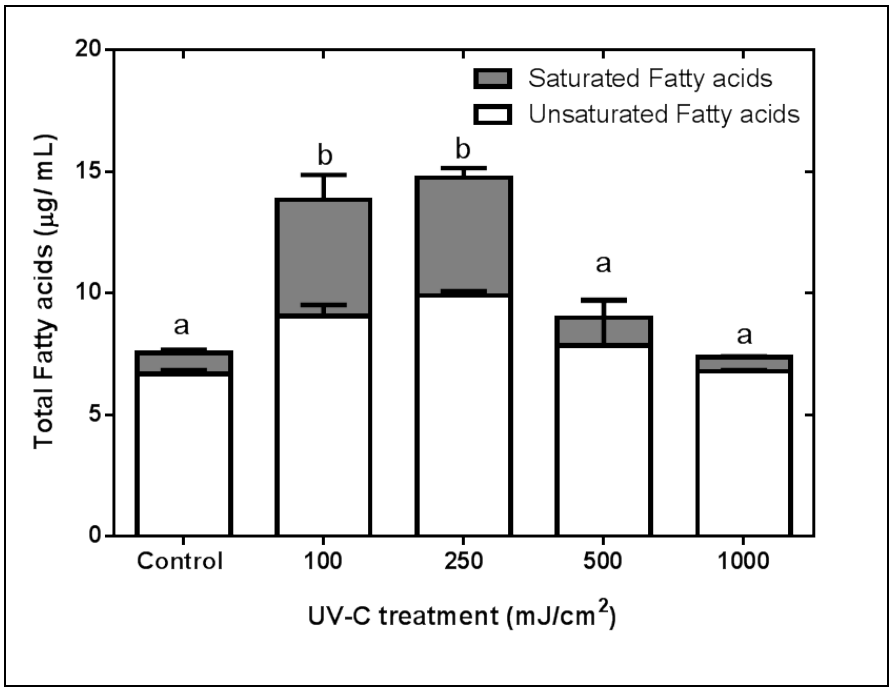


Figure 3: Total fatty acids produced by different UV-C-treated *Chlorella* sp. BR2 cultures. Values are mean ± SE from three separately grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).

Discussion

This study firstly introduced UV-C radiation to induce lipids production in *Chlorella* sp. concomitant with lipid induction; the algal cells were also relatively altered. While the cell size gradually increased with higher UV-C dosages, the cell survival rate significantly declined. Out of the different UV-C intensities tested, the maximum lipid fluorescence was displayed by cultures radiated at 500 mJ/cm^2 (Fig. 2c). However, the presence of free lipid bodies from ruptured cells at higher UV-C dosages (Fig. 1a) rather than intracellular lipids probably contributed to this. This is also suggested by the TAG data obtained from TLC (Online Resource Fig. 1) and GC-MS data on total fatty acids (Fig. 3) where UV-C dosages of 100 and 250 mJ/cm^2 led to the highest induction compared to the untreated control cells. GC-MS data further revealed that the UV-C-based lipid induction led to both, an increase in saturated and unsaturated fatty acids, especially at 100 and 250 mJ/cm^2. (Fig. 3), although to a different extend for individual fatty acids (Online Resource Fig. 3 and 4). This study highlights the efficiency of UV-C radiation on rapid microalgae lipid induction on actively-dividing *Chlorella* cells, and also provides a rapid method to analysis lipid by FACS. Compared to lipid induction by conventional cultivation and stress stimulations, e.g. by gradual nutrient depletion [9,37], UV-C radiation only take a few seconds or minutes and lipid stimulation could be obtained within 24 hours. It should be investigated whether UV-C radiation may serve as a viable alternative to achieve higher lipid productivities in large-scale cultivation systems where cells can remain in exponential growth phase, in comparison to cells that have to undergo lengthy nutrient depletion phases.

Coincident with many studies conducted with UV-B radiation [e.g. 30,31], an increase in cell size was also found in this study. Similarly, a study on nitrogen deprivation in the marine microalga *Dunaliella tertiolecta* also noted an increase in cell size with more lipid accumulation [32]. So it was implied that the cell size may have increased by UV-C as a result of lipid induction. However, larger cells could be better adapted to tolerate UV radiation as suggested by previous UV-B research [30,31]. Since UV can cause genetic mutations on most life forms [18,33], UV may have acted as a selection pressure during algal evolution [34,35].

Along with the increase of cell size, the cell survival rate declined dramatically in this study and some of the cells had burst open and lipid bodies were released in medium (Fig. 1a). This emphasizes the need to carefully optimize UV-C dosages to achieve maximum lipid

induction (and lipid productivity) for the culture. However, a pre-treatment with UV-C that leads to breaking of the cells may have a positive effect on lipid extraction efficiency.

In this study, the P1 population in FACS analyses presented the auto-fluorescence of algal cells, while P2 was attributed to lipid fluorescence (Online Resource Fig. 2). The conversion of cells from P1 to P2 was a clear sign of lipid stimulation by UV-C radiation. The lipid stimulation in *Chlorella* sp. BR2 was closely correlated to the amount of P2 population between 100 and 500 mJ/cm^2, where the average lipid content in P2 cells also increased significantly (Fig. 2). Although the total lipid content increased by UV-C radiation at different extents, an alteration of fatty acid profiles was significant at 100 and 250 mJ/cm^2 (Fig. 3; Online Resource Fig. 3 and 4). Interestingly, the decrease of C16, C18 and C20 SFAs corresponded to the increase of C16:2, C16:3, C18:1, C18:2, C18:3 and C20:4 USFAs (Fig. 4). A similar result was also obtained for *Spirulina platensis* with UV-B radiation, concomitant with deleterious effect on thylakoid membrane integrity and protein profile [36].

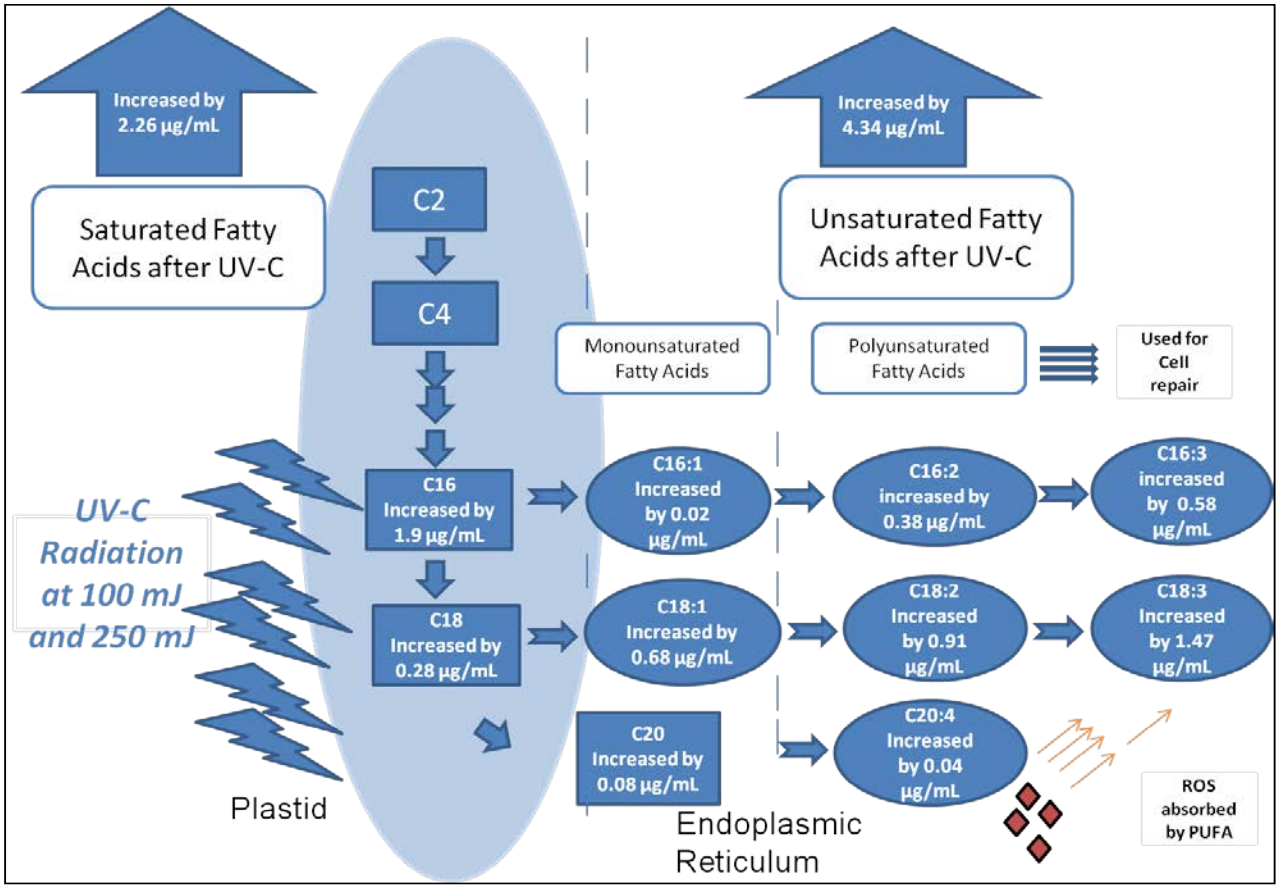


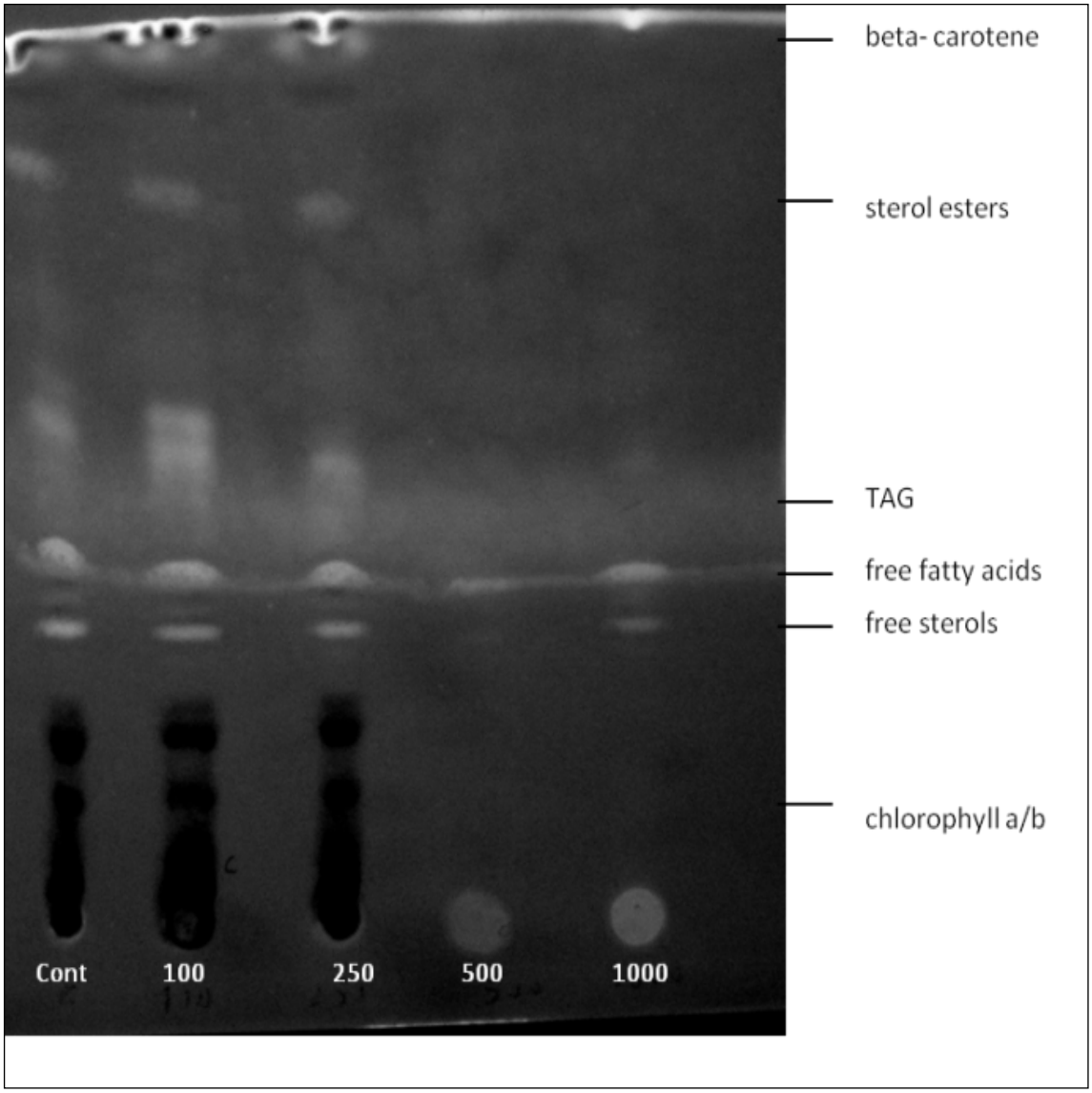
Figure 4: Effect of low dosage of UV-C radiation on the fatty acid synthesis pathway. Energy obtained from UV-C radiation might help in conversion of saturated fatty acids that

serve as a storage function in plastids to unsaturated fatty acids that serve as antioxidants and repair damage to membrane lipids.

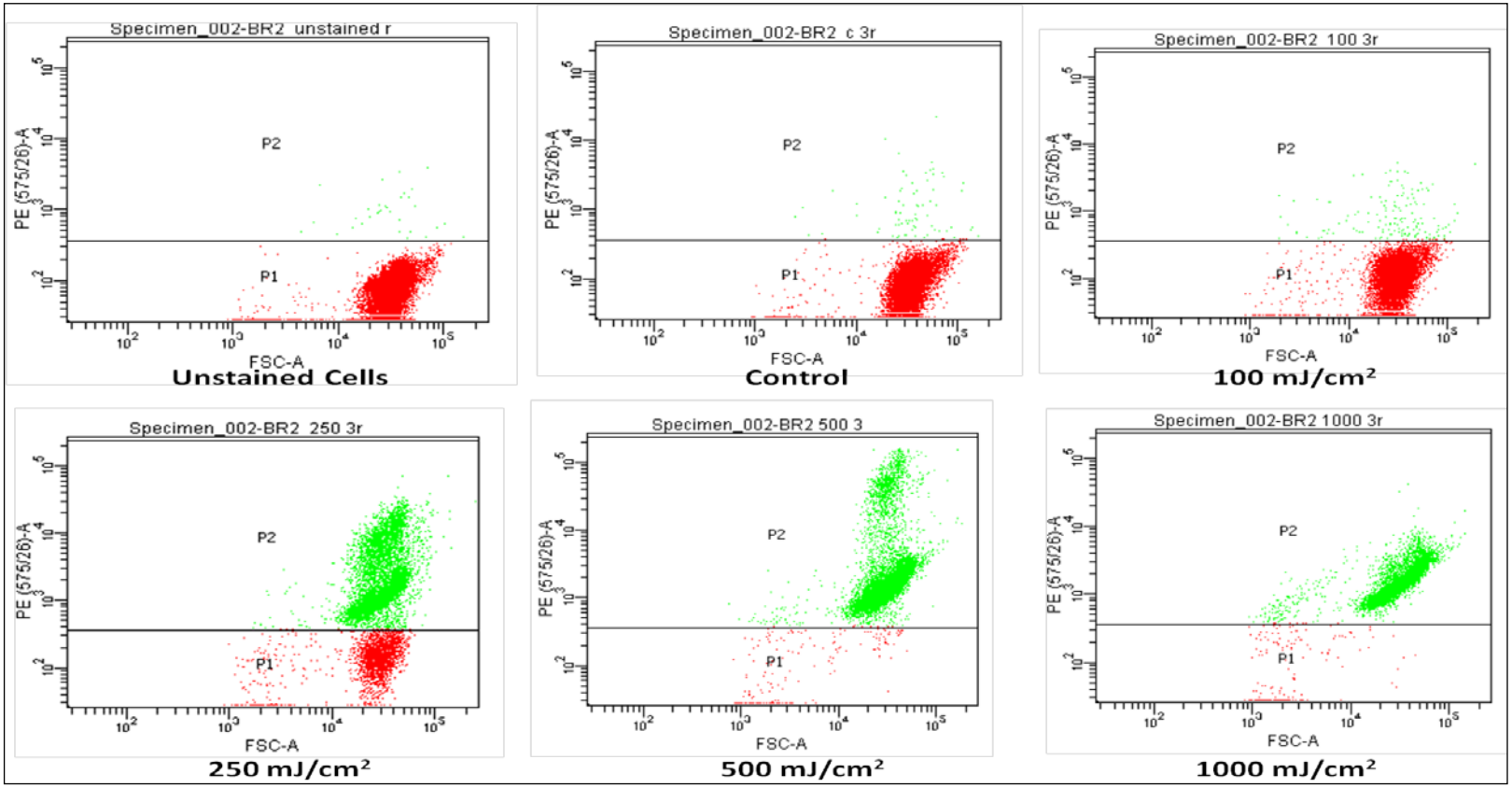
Amongst these USFAs, the increment of PUFAs was the main change in this study. As PUFAs are involved in cell repair and growth [38,39], it is conceivable that *Chlorella* sp. BR2 cells were repairing the photo-damage caused by UV-C. However, it seems that the damage was irreversible when algal cells were exposed to high UV-C radiation since the lipid profile did not change in 24 hours at 500 and 1000 mJ/cm^2 of UV-C radiation (Online Resource Fig. 3 and 4)

Fatty acid desaturation inserts double bond(s) into pre-formed fatty acid chains [40]. Therefore, it is tempting to speculate that the increase of C16:2, C16:3, C18:1, C18:2, C18:3 and C20:4 USFAs were actually the products of the desaturation of C16, C18 and C 20 SFAs in *Chlorella* sp. BR2 (Fig 4). Thus it can be concluded that lower UV-C dosage (i.e. 100 and 250 mJ/cm^2) may help to convert SFAs to USFAs with a significant increase in PUFAs within 24 h after treatment.

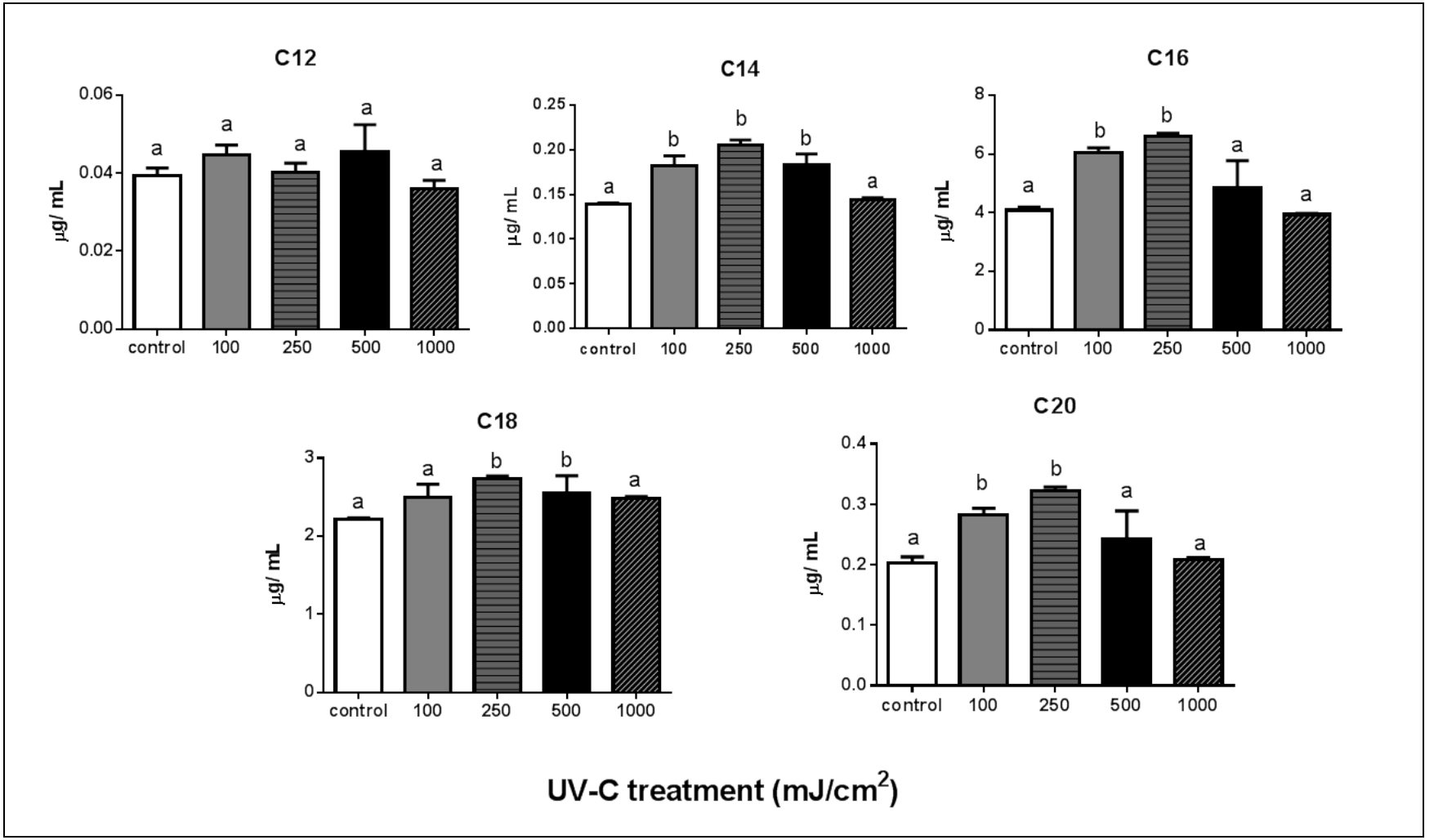
In microalgae, the deleterious effect of UV light on thylakoid membrane integrity and protein profile is accompanied by the generation of reactive oxygen species (ROS) [36,41]. It has been proven that PUFAs have a strong affinity or absorption to ROS [41]. Therefore, the increment of PUFAs may present a protection mechanism of algal cells to ROS, while membrane damage due to lipid peroxidation would also create the need to repair membrane damage with polar lipids containing PUFAs.



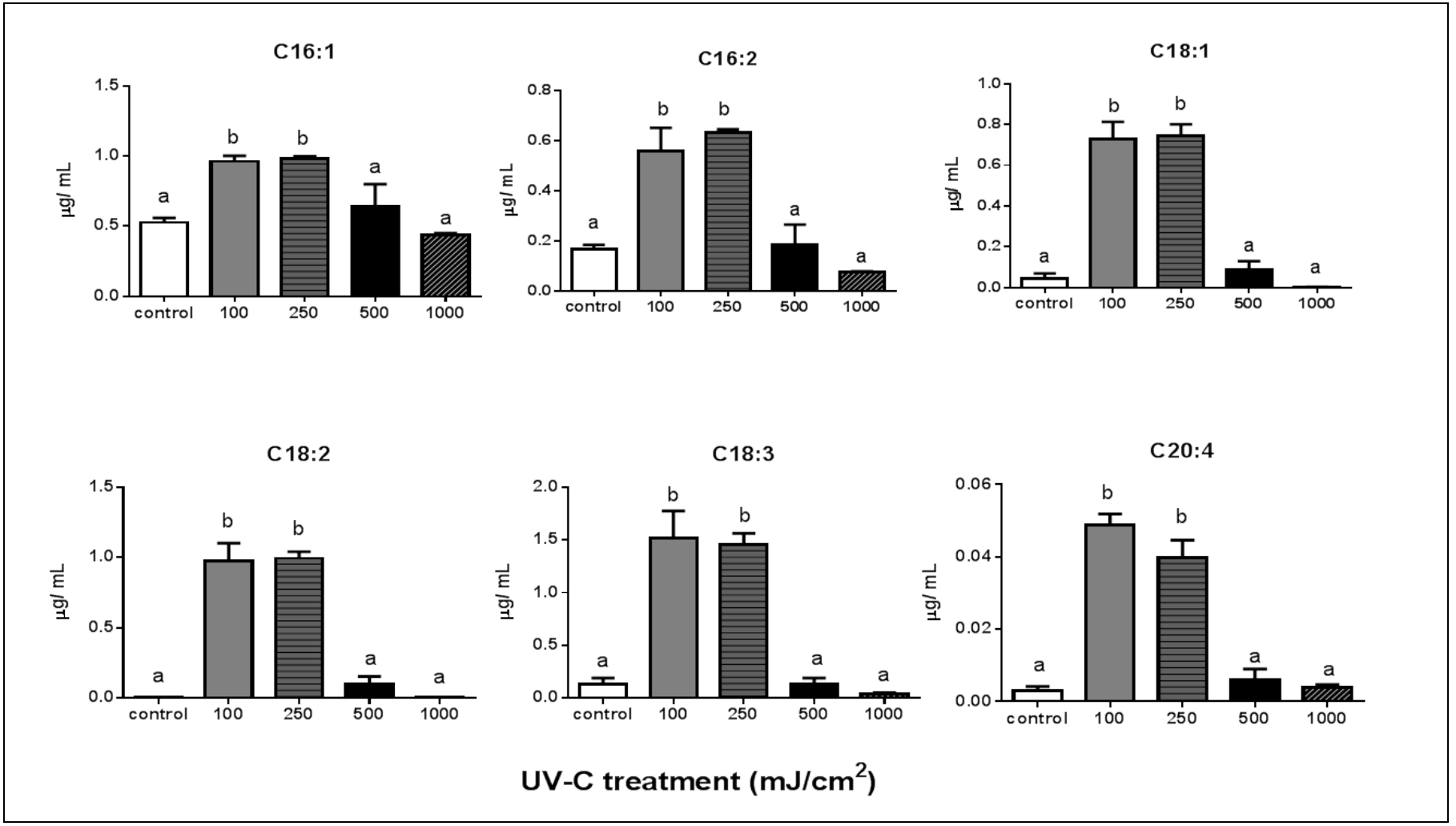
Online Resource Figure 1: Analysis of the non-polar fraction of TAGs in C*hlorella* sp. BR2 treated with different UV-C radiation doses (0-1000 mJ/cm^2).



Online Resource Figure 2: FACS analysis of *Chlorella* sp. BR2. Shown are cells without Nile red staining (Unstained Cells) and Nile red-stained with different UV-C dosages ranging from 0 mJ/cm^2 (Control) to 1000 mJ/cm^2 showing P1 and P2 populations. The Y-axis shows fluorescence intensity at the phycoerythrin excitation wavelength of 575 nm and the X-axis shows the forward scatter based on cell size.



Online Resource Figure 3: Comparison of different saturated fatty acids present in *Chlorella* sp. BR2 cultures treated with different doses of UV-C radiation. Values are mean ± SE from three separately-grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).



Online Resource Figure 4: Comparison of different unsaturated fatty acids present in *Chlorella* sp. BR2 cultures treated with different doses of UV-C radiation. Values are mean ± SE from three separately-grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).

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Chapter 4: UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production

Overview

Preliminary experiments using UV-C radiation on C*hlorella* sp. BR2 indicated that this external stress can also stimulate lipid accumulation. To test whether both, nutrient starvation and UV-C treatment can lead to further lipid biosynthesis and facilitate settling, combined sequential stress treatments were carried out on *Tetraselmis* sp. (M8)., and was optimized to be completed within 48 h using pilot-scale outdoor cultivation. This chapter comprises the main findings of this thesis and involves the development of several new approaches to achieve practical solutions for microalgae cultivation, lipid induction and microalgae harvesting.

Key Findings

- This process resulted in a significant increase of both volumetric and areal lipid productivity with higher polyunsaturated fatty acids contents, while considerably reducing harvesting costs.
- Other benefits include control of co-cultured microbes and sanitized water for recirculation.
- UV-C-mediated lipid induction and settling (LIS) may contribute to commercial microalgal biofuel production.

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Chapter 4: UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production

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Abstract

Microalgae are highly efficient primary producers that can be grown in most types of water on non-arable land as a promising source of biofuel. However, large-scale microalgal biofuel production is currently uneconomical due to slow growth of high-percentage oleaginous algae and large harvesting costs. Here we present a new strategy, using a small dose of externally applied UV-C radiation, that significantly increases lipid contents of fast growing microalgae and that at higher doses also results in rapid settling. The procedure essentially separates biomass growth from lipid accumulation and harvesting which was optimized to be completed within 48 h for *Tetraselmis* sp. This process resulted in a significant increase of both volumetric and areal lipid productivity with higher polyunsaturated fatty acids contents, while considerably reducing harvesting costs. Other benefits include control of co-cultured microbes and sanitized water for recirculation. UV-C-mediated lipid induction and settling (LIS) may contribute to commercial microalgal biofuel production.

Broader context

Biofuel production by microalgae is considered a promising approach but that is currently uneconomical due to slow-growing high-lipid producing microalgal strains and high harvesting costs. The first problem is essentially that microalgae cannot grow rapidly and produce large amounts of lipids simultaneously. The second problem is that microalgae are microscopic organisms that are currently mostly harvested by centrifugation, a very expensive and energy-intensive step that is not easily scalable. Here we present a new

approach that addresses both problems. UV-C radiation was used as an easily-applied external stimulant to rapidly induce lipids and also as a new method to induce overnight settling of microalgae for harvesting (a step that would normally require expensive flocculants or centrifuges).

Introduction

With growing tension on arable land and irrigation water resources, the case for algae as future food source, CO_2 sequestration medium, and biofuel supply becomes more compelling [1-4]. New technologies need to produce an additional 5-6 billion tons of organic carbon apart from agricultural crops [5]. Large-scale microalgae cultivation is considered one of the most promising feedstocks to produce biofuels without competing for food production or biodiverse natural landscapes; recent progress and future perspectives have been intensively reviewed [3-7]. Microalgae can also be cultivated in brackish or seawater and as an integrated concept with wastewater treatment to optimize the energetic, nutrient and financial input for feedstock production [8]. However, high capital costs, low lipid productivity of fast-growing microalgae and high harvesting & processing costs are major bottlenecks, hindering commercial production of microalgal oil-derived biodiesel. To obtain high-lipid contents in fast-growing microalgae, typically, lipid induction techniques need to be applied, but these may slow down growth and add extra costs [9]. Harvesting is another critical and challenging stage in mass microalgae cultivation due to low biomass concentrations (typically 0.3-0.5 g dry weight (DW) L^{-1}) [10, 11], large volumes of water, and small cell sizes [12-16]. Harvesting consumes 20-30% of biomass production costs [13, 17, 18], is alga-specific and may comprise different physical, chemical and biological processes [11, 12, 18, 19].

Synthesis and accumulation of large amounts of triacylglycerides (TAG) in microalgae is required for biodiesel production by transesterification. Cellular lipid and TAG induction techniques (either acting individually or in combination include nutrient stress, osmotic stress, radiation, pH and temperature change, heavy metals and other chemicals, and some metabolic engineering approaches [9, 20-26]. Nitrate starvation is most widely-studied in almost all candidate biofuel microalgae [9] and is easily-applied by omitting nitrate in the growth medium or letting the culture use up nutrients. However, it typically takes 3-5 days until significant amounts of lipids are synthesized which is accompanied by slow growth rates and thus finally affects total biomass and lipid productivity [27]. Change in temperature, pH, salinity and heavy metals are difficult to regulate at large-scale. Genetically-modified

microalgae potentially produce more lipids, but regulatory issues increase costs, while microalgae naturally high in lipids or hydrocarbons are typically slow-growing [28].

Thus there is demand for a process that can not only rapidly induce lipids in exponentially growing algae, but also bridge the gap between the time taken in microalgal biomass production to harvesting the biomass. After considering different lipid induction techniques, we concluded that, external lipid induction with light irradiation is a promising approach that does not involve any alternation in growth media, or leave traces in the biomass and that, moreover, can be easily dosed. Solar ultraviolet light comprises UV-A (400-315 nm; 3.10–3.94 eV/photon), UV-B (315-280 nm; 3.94–4.43 eV/photon), and UV-C (280-100 nm; 4.43–12.4 eV/photon), but the latter does not reach the Earth's surface. UV-A and UV-B have already been tested for microalgae, but lipid induction took more than 2-3 days to show an effect [22, 24, 29-31]. As UV-C light carries more energy per photon, the hypotheses was to use UV-C (253 nm) radiation as a stress induction technique after reaching a certain cell density, to minimize biomass loss and obtain high-lipid productivity. Our results demonstrate that UV-C stress not only led to doubling of cellular lipid contents but also the loss of flagella and subsequent settling, a convenient, time-saving and cost-effective way for microalgae harvesting.

Methods

Laboratory-scale microalgae culturing and UV-C treatment

Strain *Tetraselmis* sp. M8 was collected in an intertidal rock pool at Maroochydore, Australia (26°39'39"S 153°6,18"E; 12 pm on 6 August 2009 [32]). Pure cultures of isolate M8 were grown in f/2 medium [48] in autoclaved artificial sea water (35 ppt NaCl). Laboratory culturing conditions were set at 23°C on an orbital shaker (Thermoline) at 100 rpm with 12 hours of light and dark rhythm. When the cell density reached 10^6/mL, the culture was used for UV-C radiation trials. Primary stock cultures were maintained aerobically in 100 mL Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent white light (120 µmol photons $m^{-2}s^{-1}$). Nitrate and phosphate levels were determined using the corresponding API Nutrient testing kit according to the manufacturer's instructions. After gently stirring, 5 mL aliquots of M8 culture were pipetted into a Petri dish, forming a thin layer inside (total of 20 plates). Plates were randomly divided into five groups with three plates used for each for UV-C radiation (253 nm)). with dimension of 31.7 x 24.1 x 15.2 cm containing five G8T5 format, minibipin

bulbs (Biorad, Gs-Genelinker, California, USA). They were separately irradiated at 0, 100, 250, 500 and 1000 mJ/cm^2 (1 J/s= 1 Watt). They were separately irradiated at 0, 100, 250, 500 and 1000 mJ/cm^2. All Petri dish cultures were subsequently incubated for 24 h. Then the algae survival rate was measured by counting the live cells based on visibly intact chloroplasts in each replicate. The cell size was also measured by compound microscopy (Olympus).

Lipid fluorescence analysis

For each replicate, 1 mL of algae cells was sampled and stained with 3 µL Nile Red solution (a 10 mg/mL Nile Red (Sigma, USA) stock solution was prepared in acetone and stored in the dark at 4°C). After 20 min incubation in the dark, the lipid fluorescence intensity of cells was detected by fluorescence-activated cell analyzing (BD LSR II: Analyzing flow cytometer) with 573 nm of excitation wavelength. A total of 10,000 cells were counted in each sample. A gate was set up to separate the fluorescence- activated cells and inactivated cells based on the analysis of cells without Nile Red staining. The background absorbance from chloroplast auto-fluorescence was subtracted from each sample using mock-treated (control) *Tetraselmis* sp. M8. Qualitative observation was conducted simultaneously by fluorescence microscopy as described below. For plate reader analyses samples were stained with Nile red stock solution as mention above and 200 µL of each sample was loaded into 96 well-plates and analyzed with a Fluostar optima and Polarstar optima (BMG LAB tech) plate reader at excitation and emission wavelength of 485/584 nm; the gain was set at 2400.

GC-MS analyses

GC-MS analysis was carried out as described previously [32]. Briefly, 4 mL of algal culture was centrifuged at 16,000xg for 3 min. The supernatant was discarded and the lipid in the algal pellet was hydrolyzed and methyl-esterified with 300 µL of 2% H_2SO_4 methanol solution at 80°C by shaking (480 rpm) for 2 h on a thermalmixturer (Eppendorff). Prior to esterification, 50 µg of heneicosanoic acid (C21) was added to the pellet in each sample as an internal standard (IS). After esterification, 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane (analytical grade) were added and vortexed for 20 s. Phase separation was performed by centrifugation at 16,000xg for 3 min and the hexane layer was used for lipid profile analysis. GC/MS analyses were carried out on an Agilent 6890 GC coupled to a 5975 MSD. A DB-Wax column (Agilent, 122-7032) was used with running conditions as described in Agilent's RTL DBWax method (Application note: 5988-5871EN). Identification

of fatty acid methyl esters was based on mass spectral profiles and retention times in the Agilent's RTL DBWax method. Each fatty acid methyl ester (FAME) was quantified using the formula:

Fatty acid (µg/mL) = (Total ionic current of fatty acid/ Total ionic current of standard) x (molecular mass of fatty acid/molecular mass of internal standard) x 50/4

Enzymatic assays

Lipid peroxidation, superoxide dismutase (SOD) and glutathione reductase (GR) activities were determined using the TBARS, Superoxide Dismutase or Glutathione Reductase (GR) Assay Kits, respectively, (Cayman) according to the manufacturer's instructions. A total of 5 mL culture with 10^6 cells/mL was used as UV-C treated cells (0–1000 mJ/cm^2) and a mock-treated control culture were centrifuged at 2000xg and resuspended in 1 mL diluted assay buffer provided by the manufacturer. The cultures were then sonicated three times for 5 min at 40 V setting on ice, centrifuged at 1500xg for five min at 4°C and the supernatant was used to perform the assay.

UV-C-mediated microalgal lipid biosynthesis induction and settling (LIS)

A total of 20 L of exponentially-grown culture of *Tetraselmis* sp. M8 were taken from a 1000 L raceway pond and poured in two 50 L-aquarium fish tanks, each containing 10 L of culture (Supplementary Fig. 5). One of the tanks was UV-C-radiated for 15 min (3 J/cm^2) with a custom-built UV-C chamber with continuous bubbling to ensure that all cells were evenly radiated, while the other tank was a mock-treated control culture without UV-C radiation that was bubbled for the same time. A total of 5 mL of the culture from both the tanks was taken into 20 mL Falcon tubes for Nile red-staining and cell counting. The culture was left overnight for settling and lipid induction. Cell count was carried out at 3 h and 15 h after treatment.

Outdoor raceway cultivation

In order to evaluate the UV-C lipid induction and settling technique of microalgae in a mid-scale outdoor setting, 20 L of laboratory-grown *Tetraselmis* sp. M8 culture was used to inoculate two identical 1000 L outdoor raceway ponds built by The University of Queensland's Algae Biotechnology Laboratory (www.algaebiotech.org; Supplementary Fig. 7). Mid-scale outdoor raceway experiments were conducted between January and

September 2012, with most experiments carried out during winter time under sunny conditions when average day temperatures ranged from 22°C–26.5°C. Cultures were continuously grown in 1000 L-outdoor raceway ponds and climatically adapted before experiments commenced. Cultures were continuously grown in seawater containing f/2 medium at uncontrolled pH values of 8.8-9.1 and cell culture densities of 1.5-2.3×10^6/mL. Cell counts were conducted daily followed by Nile red fluorescence and GC-MS analyses during controlled experiments. Cultures were also checked daily under the microscope to ensure that no contamination with other microalgae occurred.

After initial optimization experiments in the outdoor raceway ponds, 500 L of microalgae culture with 12 cm water depth was used for 6 h (72 J/cm^2) of UV-C treatment. This dosage was found suitable to induce lipid biosynthesis with minimum cell death. Subsequent experiments were set up for 8 days out of which typically by day 3 and 4 the culture was under N starvation and after which an increase in fluorescence of Nile red-stained cells could be measured. To further minimize initial cell mortality, the UV-C dosage was divided into two phases. An initial dose of UV-C was applied on day 5 for 4 h followed by 36 h continued cultivation for rapid lipid induction. A final dose of UV-C was applied for 2 h at the end of day 7, culture-mixing was stopped and the culture was harvested on the next morning after overnight settling. The experiment was repeated three times and included a raceway swap, but included a reduced frequency of measurements.

Dry weight measurement

At the end of outdoor cultivation, just before harvesting, 25 mL of the culture was used for dry weight measurements. The 25 mL culture was filtered through a 0.27 µm glass fiber filter (Millipore) which was pre-weighed and pre-washed with 1 mL distilled water in a vacuumed filter unit (three biological replicates were used from each culture (UV-C treated and mock-treated control)). After filtration the filters were kept in individual Petri dishes to avoid contamination and dried in a drying oven for 24 h at 80°C with the plate lid half open. After 24 h the filters were immediately weighted.
To determine the dry weight the following formula was used:

$$\text{Dry weight mg/L} = \frac{\text{Filter dry weight - Filter pre-weight}}{\text{Filtered volume mL x 1000}}$$

Dry weight in mg/L was determined from the average of three weight measurements for each replicate.

Microscopic analyses

After a lipid induction phase, microalgae cells were stained with 2 µg/mL Nile red (dissolved in acetone; Sigma, USA) for 15 min and photographed using a fluorescent Olympus BX61 microscope fitted with a 100 W High Pressure Mercury Burner and an Olympus DP10 digital camera. Differential interference contrast (DIC) and epifluorescent (excitation: 510–550 nm, emission: 590 nm) images were obtained at 1000× magnification with oil immersion.

UV-C disinfectant effect

On the morning on day eight of the experiment, just before harvesting, the top layer of both the raceways was collected and 100 µL of the culture was spread on LB plates and PDB plates to screen for bacteria ad fungal colonies present after the experiment. The bacterial plates were incubated in a 37°C growth chamber for 48 h, whereas fungal plates were incubated for 7 days at room temperature. Microbial growth was monitored every 24 h.

Analytical methods

Measurement of nitrate and phosphate levels in the photobioreactor was performed using colorimetric assays (API, Aquarium Pharmaceuticals and Nutrafin, respectively). Data for growth rates and lipid productivity was statistically analyzed by one-way analysis of variance (ANOVA) with different microalgal cultures as the source of variance and growth rate or lipid productivity as dependent variables. This was followed by Turkey's multiple comparisons test ($P<0.05$) where appropriate. Student's t-test was used for pairwise comparisons.

Construction of UV-C chamber

For scaling up of the UV-C lipid induction and settling technique, a UV-C chamber was custom-built using a stainless steel hood of a plant growth chamber light lamp in which the original fluorescent tubes were replaced with six G8T5 (TUV 8W) GE Philips Bi-Pin 288, UV-C (253 nm) lamps connected in series (Supplementary Fig. 5A). The height, length and width of the chamber was 40 cm, 45 cm and 40 cm, respectively. Based on a comparison to the specifications of the laboratory UV chamber (BioRAD, Gs-Genelinker), the heat losses of the lamps and the set-up of the outdoor chamber, 12.5% of the energy

was estimated to reach the culture surface as UV-C radiation (approx. 12 J/cm^{-2} h^{-1}; actual UV-C surface radiation was not measured).

Techno-economic analysis

Using the time taken to harvest 1,000 L of *Tetraselmis* sp. M8 culture in mid-scale outdoor cultivation and information from previously carried out studies and specifications provided by companies that supply the equipment and chemicals, a theoretical calculation was carried out to determine the techno-economic feasibility of overall biomass recovery in a one-step as well as a two-steps method. For the costing purpose, harvesting of 10,000 L of *Tetraselmis* culture was considered (Table 1). Assessments were independently developed in accordance with Australian conditions and where possible, were compared to equivalent costing from previous economic analyses of microalgae biofuel systems [11].

Results

UV-C–irradiation induces microalgal lipid biosynthesis

Microalgae have been suggested as a promising source of triacylglycerides for biodiesel production. *Tetraselmis* sp. M8 microalgal cells had previously been shown to accumulate significant amounts of lipids after nutrient deprivation [32]. Preliminary experiments using UV-C radiation on various microalgal cells, including *Tetraselmis* sp. M8, indicated that this external stress can also stimulate lipid accumulation (data not shown). To test whether both, nutrient starvation and UV-C treatment can lead to further lipid biosynthesis, combined sequential stress treatments were carried out. Nile red-staining of nitrogen-deprived *Tetraselmis* sp. M8 cells indicated that an additional external stress treatment by UV-C exposure, led to an increase in cell sizes and additional lipid accumulation in lipid bodies within 24 h (Fig. 1). As UV-C doses were increased from 100 mJ/cm^2 to 250 mJ/cm^2, fluorescence intensities became stronger, but at higher doses cell rupturing was observed. Interestingly, detached flagella were found at UV-C radiation doses at and above 100 mJ/cm^2 (arrow in Fig. 1a) and this could also occasionally be observed under a fluorescence microscope with blue light excitation within a few seconds. As expected, a sharp decline in cell survival rates was observed with increased UV-C radiation (Fig. 1b). The cell survival rate was approximately half (LD50) at 250 mJ/cm^2 (Fig. 2).

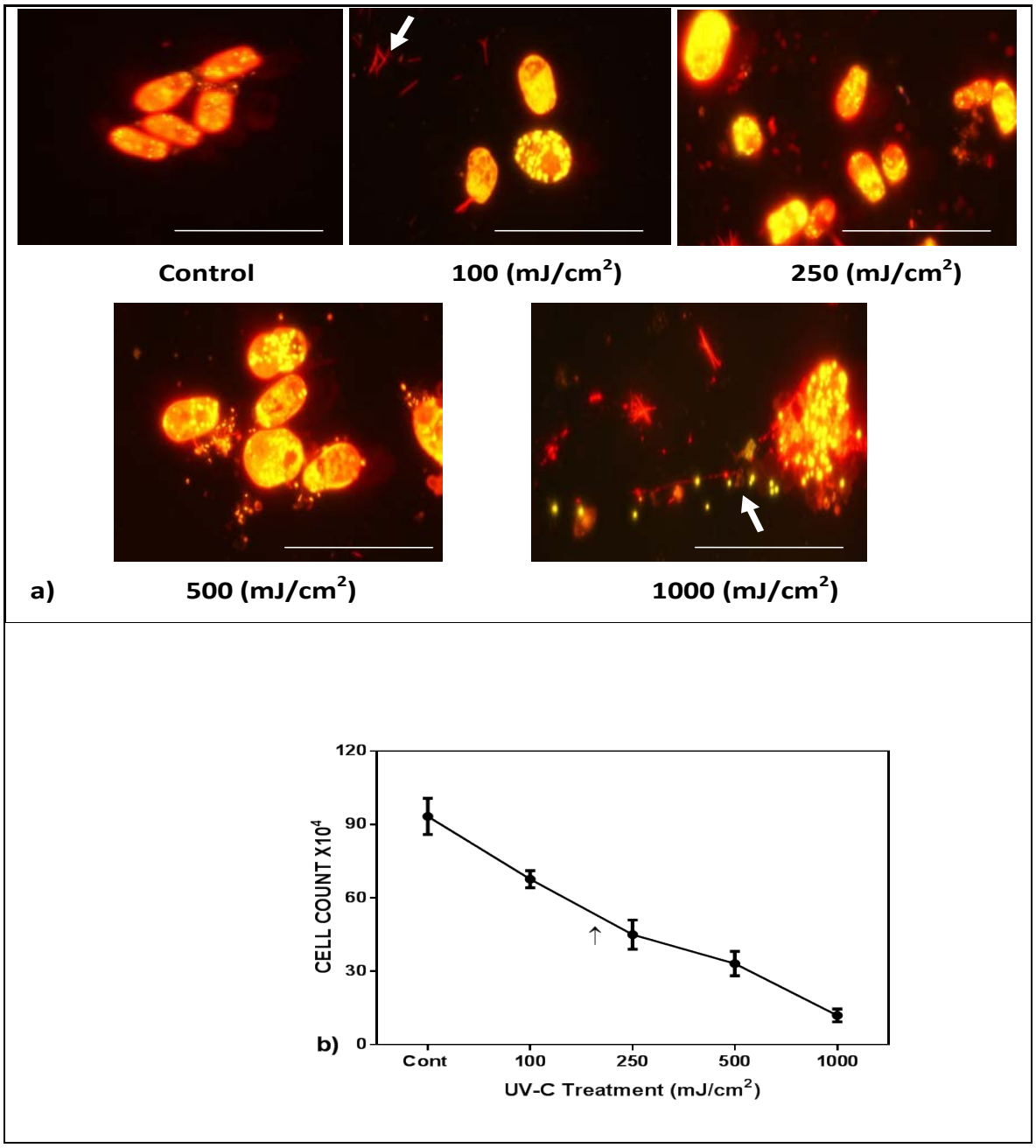

Figure 1. Nile red-stained cells of *Tetraselmis* sp. M8 that received different doses of UV-C exposure. a) Cells with maximum lipid fluorescence (yellow) can be observed at 250 mJ and 500 mJ UV-C radiation. Starting from UV-C of 100 mJ/cm^2 detached flagella can be observed (white arrow), as the radiation is increased to 1000 mJ/cm^2 cell rupturing occurs (white arrow) with lipid bodies released in the medium. Cells are shown at 40x magnification (bar=50 µm) at 24 h after treatment. b) Kill curve of *Tetraselmis* sp. M8, showing the number of cells that survived UV-C treatment at different dosages. Shown are mean values ± SEs from three independent treatments of M8 culture. The arrow indicates the LD 50 value.

To profile and quantify lipid accumulation of millions of individual nutrient-starved *Tetraselmis* cells simultaneously following different UV-C exposure, a flow cytometer was used (Fig. 2). With an increase of UV-C radiation, algal cells were divided into two different populations, termed P1 and P2, according to a background fluorescence intensity cutoff in the unstained control population. The majority of the P1 population in this control can be attributed to auto-fluorescence of chloroplasts (Supplementary Fig. 1a). In the untreated, but Nile red-stained control, 60% of the cells were allocated to the P1 population, whereas 40% were in the P2 population (Fig. 2a; Supplementary Fig. 1b); the latter can be attributed to lipid accumulation by nutrient deprivation in these cells. When UV-C radiation of 100 or 250 mJ/cm^2 was used for treatment of nutrient-starved cells, P1 was reduced to 40%, while the lipid-accumulating P2 increased to 60%, indicating a marked increase in lipid production after UV-C treatment (Fig. 2a; Supplementary Fig. 1c,d). However, at 500 mJ/cm^2 P1 and P2 were equal and at 1000 mJ/cm^2 P1 further increased to 65% while the high lipid-containing P2 decreased to 35% (Fig. 2A; Supplementary Fig. 1e,f). This is consistent with microscopic observations, suggesting that high UV-C doses damaged cells to a point where most cells were unable to produce Nile red fluorescence-detectable lipids. Quantification based on average fluorescence intensities from three separately-grown and -treated microalgal populations, showed that P1 cells always had much less fluorescence intensities than the lipid-accumulating P2 populations (Fig. 2b). Overall, fluorescence intensities of both populations combined, confirmed that nutrient-starved UV-C-treated cells (100 mJ/cm^2) had significantly increased lipid contents (P=0.022: Fig. 2c). However, there was no significant fluorescence increase between the untreated control and cells treated with 250 mJ/cm^2 or higher doses which can probably be attributed to cell damage leading to cell rupturing and release of lipid bodies that were not quantified by flow cytometry.

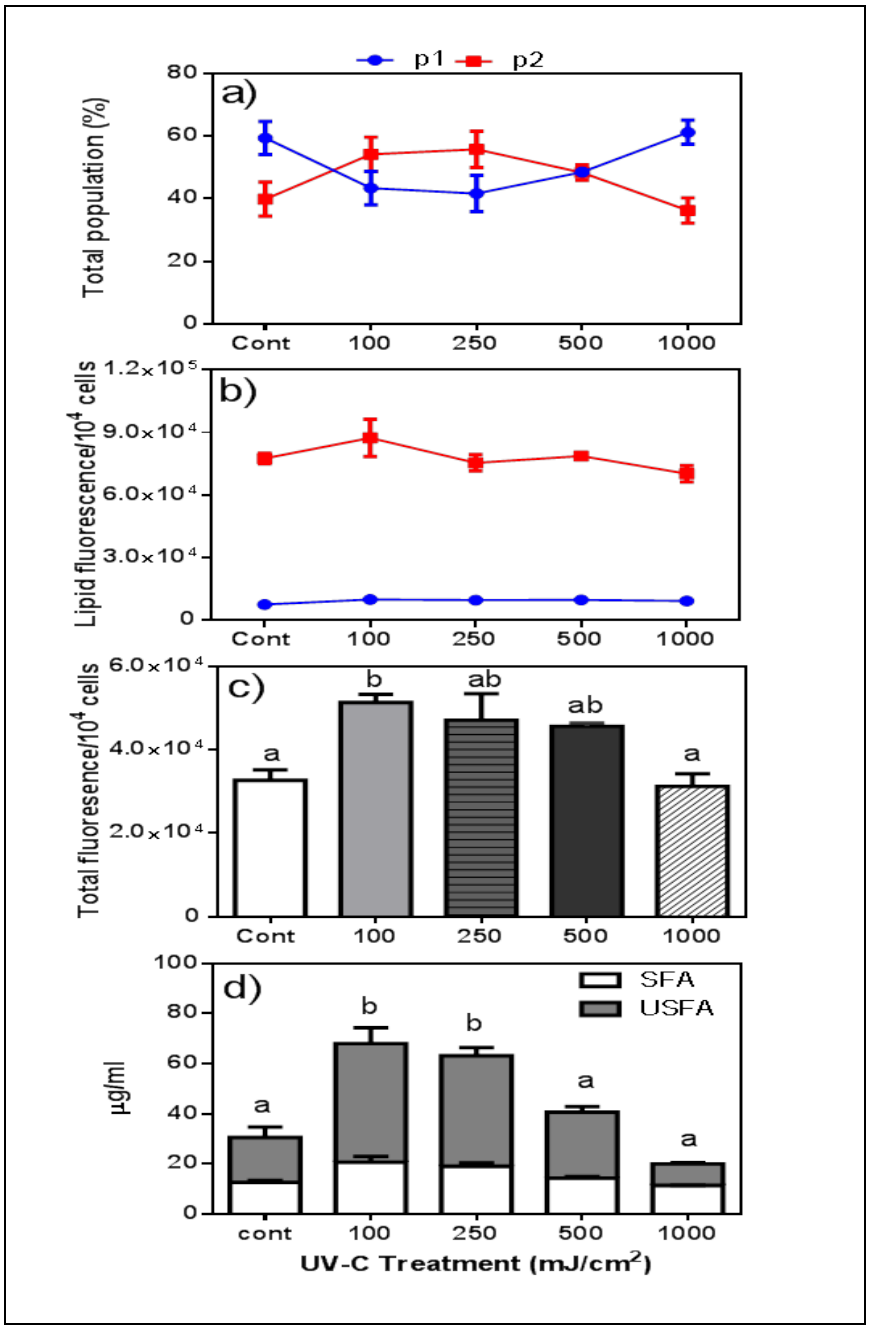

Figure 2. Lipid induction in *Tetraselmis* sp. M8 cultures at 24 h after receiving different UV-C dosages. a-c) FACS analysis of Nile red stained cells showing distribution (a) and lipid fluorescence (b) of low (P1) and high (P2) fluorescence cell populations and of the total population (c). d) Triacylglyceride quantification by gas chromatography-mass spectroscopy (GC-MS) showing total fatty acids as well as saturated (SFA) and unsaturated (USFA) fatty acids produced by different UV-C-treated cultures. Shown are mean values ± SEs from three separately-grown and treated microalgae cultures. Bars with different letters indicate significant differences (P<0.05). See Supplementary Figure 1 for the corresponding scatter plots of P1 and P2 populations and Supplementary Figures 2 and 3 for graphs of individual fatty acids of microalgae treated with different UV-C dosages.

Fatty acid profiles of UV-C-treated microalgae shift towards unsaturated fatty acids

To further quantify the ability of UV-C stress to increase cellular TAG contents and to profile the fatty acid composition, GC-MS analyses were performed. These confirmed the results obtained from flow cytometry, showing a significant total fatty acid increase (*P*=0.032, *P*=0.014; respectively) of UV-C-treated cultures (100 and 250 mJ/cm^2) compared to untreated controls, whereas cultures treated with 500 and 1000 mJ/cm^2 showed no significant difference (Fig. 2d). Moreover, in cultures treated with 100 and 250 mJ/cm^2, the amount of unsaturated fatty acids (USFA) significantly increased and also the proportion of USFA compared to total fatty acids (Fig. 2d). Amongst the saturated fatty acids (SFA), the highest increase was observed for C16 (palmitic acid), followed by C14 (Supplementary Fig. 2). On the other hand, C18 and C20 did not show any increase in cultures treated with 100 and 250 mJ/cm^2 UV-C, whereas in cultures treated with 500 and 1000 mJ/cm^2 the amount of C20 was significantly higher (*P*=0.0239, *P*=0.0164, respectively). When comparing different USFA, cultures treated with 100 and 250 mJ/cm^2 UV-C showed significant increases for all detected USFA, most notably C16:4, C18:1 cis+trans, C18:3n3 and C20:5 (Supplementary Fig. 3).

It appears plausible that UV-C stress leads to cellular lipid biosynthesis to improve survival of microalgal cells, as this has been observed for many other stresses, most of which lead to a higher proportion of SFAs [99]. To better understand the underlying mechanisms for the observed shift of fatty acids towards unsaturated fatty acids following UV-C treatment, several enzymatic assays were carried out. Decomposition of unstable peroxides derived from polyunsaturated fatty acids (PUFAs) resulting in the formation of malondialdehyde (MDA), was quantified colorimetrically following its controlled reaction with thiobarbituric acids (TBARS). Supplementary Figure 4a shows that as the UV-C radiation increased from 0-1000 mJ/cm^2 the formation of MDA increased in samples and was highest at 1000 mJ/cm^2. Similarly, superoxide dismutase (SOD) and glutathione reductase (GR) activities increased with higher UV-C doses (Supplementary Fig. 4b,c), suggesting that UV-C-treated cells underwent oxidative stress causing damage to DNA and cellular membranes. Hence the increased amount and proportion of USFA in UV-C-stressed cells may be required for repair of cellular damage to membranes and to provide sufficient antioxidant capacity to restore redox homeostasis.

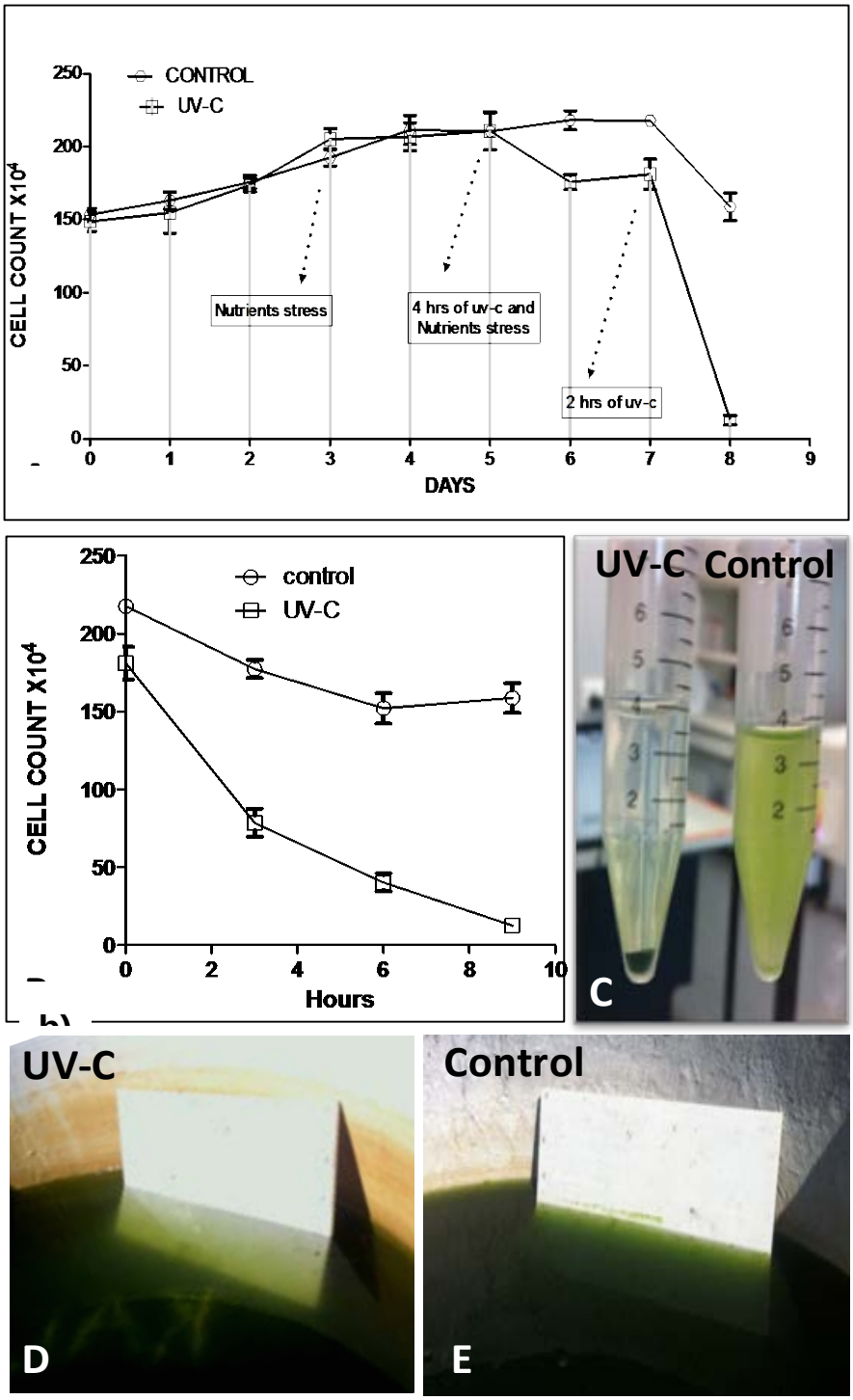

Figure 3. Settling of control and UV-C-treated algal culture in 1000-L airlift-mixed raceway ponds. a) Cell counts indicating different phases of the experiment on day 3, 5 and 7. b) Settling curve of control and UV-C-treated culture on day 8. c) Settling of UV-C-treated and control culture. d,e) Settling of UV-C-treated and control culture, respectively, in 1000-L raceway ponds against the background of a white plate. Shown for a-c are mean values ± SEs from three measurements. The experiment was repeated three times with similar results.

UV-C treatment leads to settling of flagellate algal cultures

While conducting UV-C lipid induction experiments with cultures of the flagellate microalgae *Tetraselmis* sp. M8, it was observed that cells exposed to UV-C radiation also detached their flagella and showed other signs of cell damage (Fig. 1). The effect could also be observed under blue light using fluorescence microscopy and for other flagellate microalgae (data not shown). When applied to entire laboratory-grown cultures, this resulted in settling of the algal cells if left undisturbed. Hence the notion of combining lipid induction with settling for microalgae harvesting was developed. The process of exposing flagellate algal cultures to UV-C irradiation is therefore from here-on referred to as Lipid Induction and Settling (LIS). To test whether this is applicable to outdoor conditions, algal culture settling experiments were performed on saturated 20 L-cultures grown in open-lid aquariums using a specially-designed UV-C chamber (Supplementary Fig. 5). Within 3 h of LIS treatment, about 90% cells settled when compared to untreated cultures and within 15 h >99% cells settled (Supplementary Fig. 6).

Upscaling of lipid induction and settling (LIS)

Following initial optimization of UV-C-mediated LIS to determine a suitable dose of UV-C, experiments were conducted on two pilot-scale 1000 L-raceways ponds (Supplementary Fig. 7). Both ponds were built with the exact same specifications for optimal comparisons. Cultures were first pre-adapted to growth in these ponds for 2 months where they were maintained in late exponential growth phase. For the experiment, the same culture was split into two identically-built raceways and grown simultaneously where one of the raceways was UV-C-treated and the other was mock-treated. The trials commenced at a cell density of $1.5x10^6$/mL (day 1; Fig. 3). By day 3 cells had reached the nutrient depletion point (nitrate and phosphate not measurable) and days 4 and 5 were intended for nutrient stress induction. During the early morning on day 5, an initial dose of UV-C was applied for 4 h (48 J/cm^2). As a result, the cell count dropped slightly from $1.7x10^6$ to $1.5x10^6$ in the UV-C-treated raceway (Fig. 3a). After 36 h of lipid induction phase, a final dose of UV-C was applied in the evening of day 7 for 2 h (24 J/cm^2) to induce loss of flagella and settling and Cells were left for overnight sedimentation. In the morning, more than 93% of UV-C-treated cells had settled and the remaining cell count of the culture dropped to 10^5/mL, whereas the mock-treated raceway contained $1.5x10^6$ cells/mL (Fig. 3b). Along with cell density, lipid fluorescence from Nile red-stained cells and fatty acid profiles were determined at different stages of the experiment (Fig. 4). From day 5, a clear increase in

lipid fluorescence was observed, reaching a maximum on day 6 that was twice as high in the UV-C treated raceway (Fig. 4a). As the cell density in UV-C-treated cultures was slightly lower than in the control, values for total lipids per cell were significantly higher in UV-C-treated culture on day 6 compared to the control (Fig. 4b).

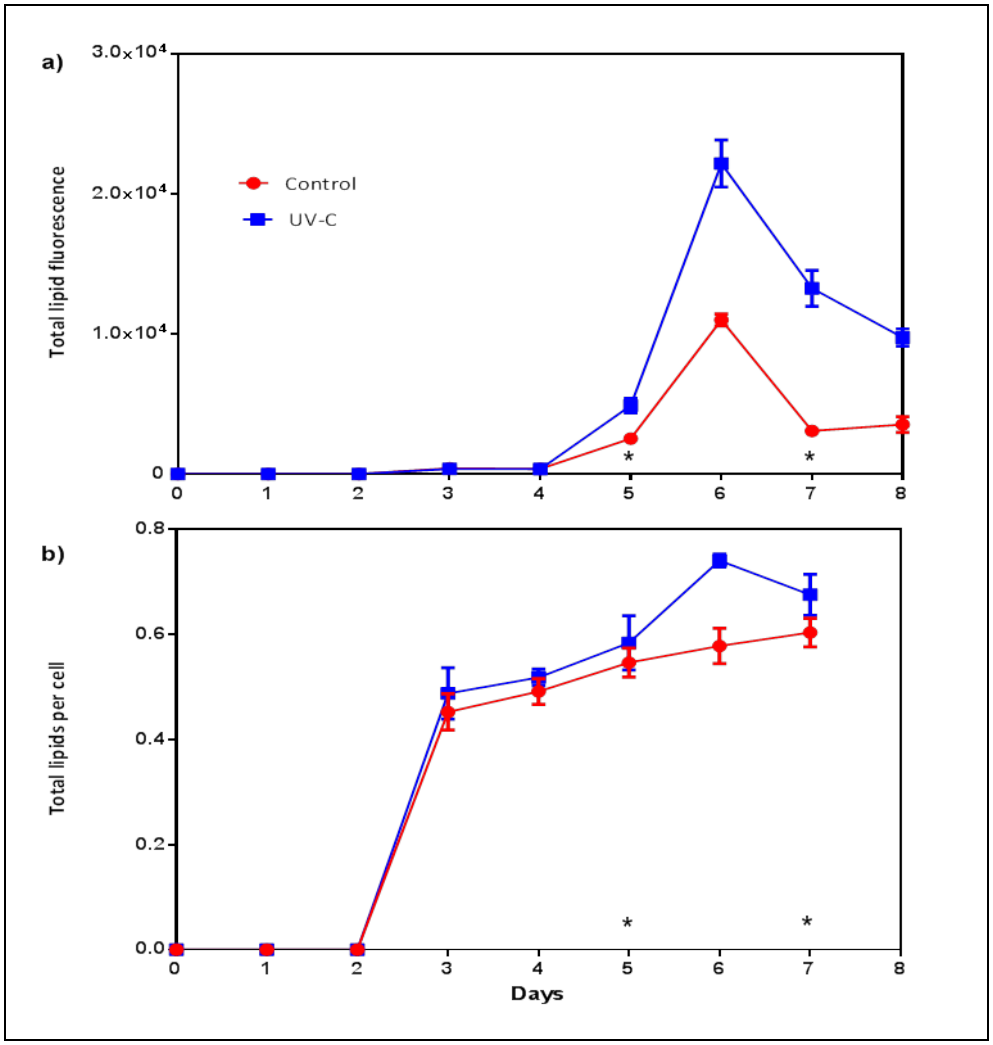

Figure 4. Lipid fluorescence of Nile red-stained cells in control and UV-C-treated raceway ponds. Maximum lipid fluorescence was observed on day 6 which was an indication of UV-C lipid induction. Shown are total culture lipid fluorescence (a) and lipid fluorescence per cell (b) as mean values ± SEs (n = 3). Asterisks indicate the time points when UV-C treatment was applied.

As expected, the total DW of harvested biomass obtained from UV-C-treated raceways was marginally reduced compared to the control (Fig. 5a). However, the total fatty acid yield, determined by GC-MS was significantly higher (300 µg/mL) when compared to the control (200 µg/mL; Fig. 5b). Consistent with the lab-scale studies, the highest increase in fatty acids could be attributed to USFA contents (P=0.028), whereas the total SFA

increase was not significant (P=0.058; Fig. 5c,d). Specifically, consistent with the lab-scale studies, the highest increases were observed for C16, C16:3, C16:4, C18:4 (Supplementary Fig. 2 and 3).

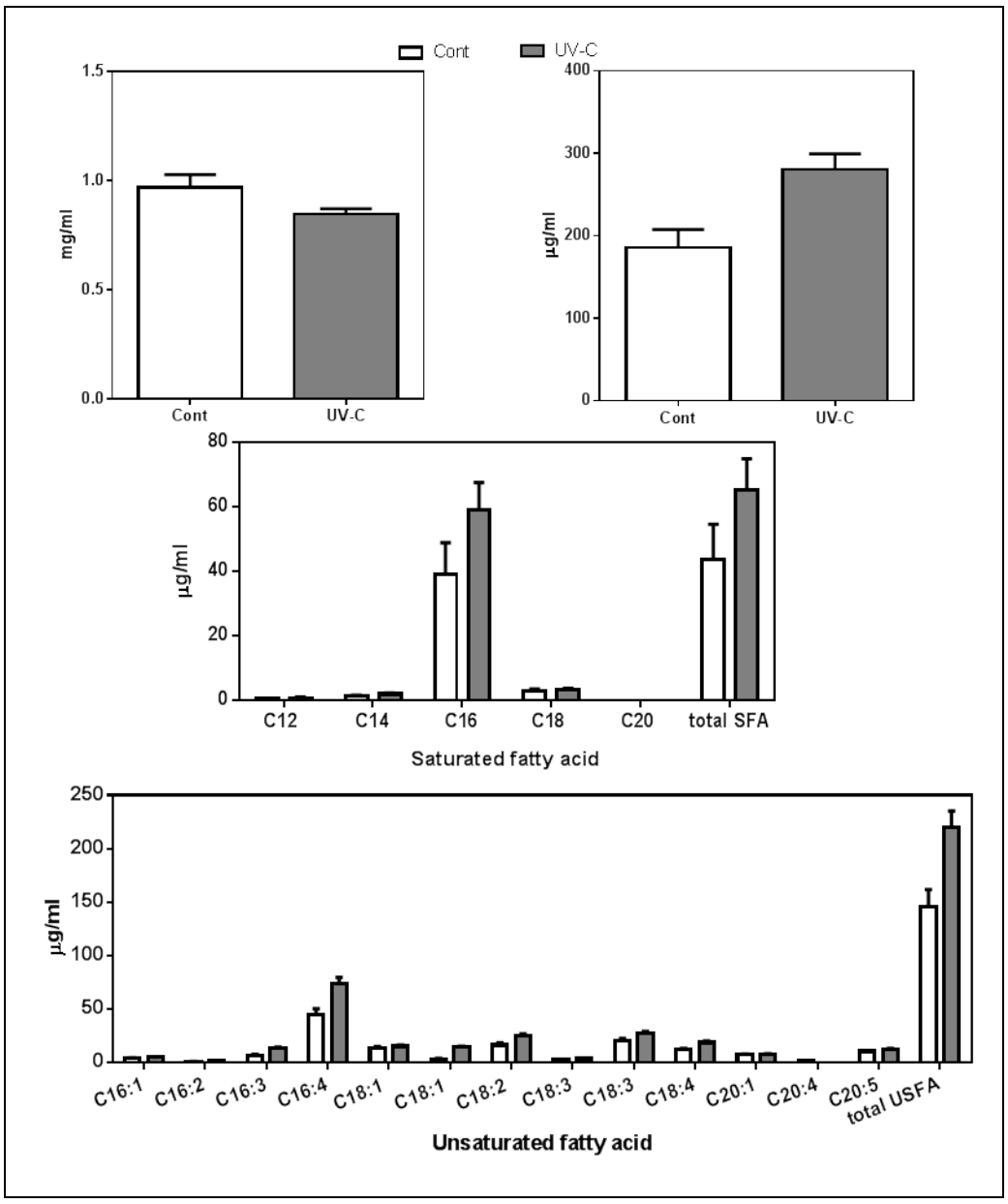

Figure 5. Biomass dry weights and fatty acid profiles in harvested culture of control and UV-C-treated raceway-grown algal cultures. Shown are total dry weights (a), total fatty acid contents (b), as well as individual and total saturated (c) and unsaturated (d) fatty acid profiles. Bars with (*) show significant differences (P<0.05). Shown are mean values ± SEs from three separately-grown and -treated raceway cultures.

UV-C treatment aids in water sanitization for reculturing

To investigate whether UV-C treatment had an effect to bacterial and fungal growth in cultures, both groups of organisms were quantified in UV-C-treated vs. untreated used

growth medium from outdoor raceway ponds. As expected, the UV-C-treated raceway pond harbored significantly-reduced culturable bacteria and fungi (Supplementary Fig. 8).

Techno-economic analysis shows reduced costs for primary dewatering

A techno-economic analysis was carried out to determine whether the implementation of LIS would result in significant cost savings compared to other currently-used methods for algal biomass harvesting. As shown in Table 1, an estimated 89% and 45% of the costs for primary dewatering of *Tetraselmis* sp. M8 can be saved for flocculant-assisted settling or dispersed-air-flotation, respectively, when using LIS instead. When considering lipid productivity rather than algal biomass, LIS stood further out as a cost-effective method as this would further reduce costs on a per lipid basis. Alternatively, cost savings maybe achieved by shortened lipid induction periods of nutrient- and UV-C-stressed cultures, compared to cultures stressed by nutrient depletion alone. Another advantage of LIS compared to flocculant-assisted methods, is that no chemical residues remain in the harvested biomass. Full life cycle analyses should be conducted on optimized large-scale cultivation systems to determine how actual costs for algal biomass and lipid production compare to other feedstocks.

Table 1. Costs of harvesting 10,000 L of *Tetraselmis* sp. culture with different harvesting techniques.

	Single Step Dewatering		**Primary Dewatering**				**Secondary Dewatering**	
	Centrifugation		**Sedimentation**	**Flotation**	**LIS UV-C**		**Filtration**	**Centrifugation**
Total Energy Consumed	55 kWh/10 m^{3}^		**-**	7.4-8.4 kWh/10 m^{3}+	5.76 kWh/10 m^{3}		1-3 kWh/m^{3}	5.5 kWh/m^{3}^
Energy Cost (AUD)$	$12.10*		**-**	$1.62 – $1.84*	$1.26*		$0.22 – $0.66*	$1.21*
Dosage required	**-**		400 g @ 40 mg L^{-1} [46]	80 g @ 8 mg L^{-1} [47]			**-**	**-**
Chemicals (AUD)	**-**		$10 (Chitosan @ $25/kg)	$0.64 (CTAB @ $8/kg)	-		**-**	**-**
pH Adjustment Dosage	**-**		1.5 to 2 L acetic acid~	**-**	-		**-**	**-**
pH Adjustment Cost	**-**		$1.20 – $1.60 @ $800/t	**-**	-		**-**	**-**
Total Costs (AUD)	**$12.10**		**$11.10 - 11.20**	**$2.26 – $2.28**	**$1.26**		**$0.22 – $0.66**	**$1.21**

$ Australian Dollar (= approx. US $1.04)

* Electricity prices were calculated based on $0.22 per kWh

^ An Evodos centrifuge was used for this study.

~ The volume was estimated by doing an experiment with 1 L of algae culture and mathematical calculation.

Values may differ for different algal strains as these may have different preferential harvesting conditions.

Discussion

This study first introduced UV-C radiation to stimulate lipid biosynthesis in microalgae. It highlights the efficiency of UV-C radiation on lipid induction and also provides a benchmark for UV-C-induced settling of flagellate microalgae. Maximum lipid induction was depicted by cultures radiated at 100 mJ/cm^2 and 250 mJ/cm^2 on Petri dishes (Fig. c,d), and 48 J/cm^2 was found suitable for 12 cm-deep *Tetraselmis* outdoor raceway cultures containing 1.5x10^6 cells/mL (Fig. 4 and 5). UV-C-induced settling occurred overnight.

Coincident with studies conducted with UV-B radiation [33, 34], an increase of cell size was found (Fig. 1a; Supplementary Fig. 1e,f). A study on nitrogen deprivation in *Dunaliella tertiolecta* also noted increased cell size following lipid accumulation [35]. So it was understood that the cell size increased by UV-C as a result of lipid induction. However, larger cells may display higher UV stress tolerance as suggested by UV-B research [33, 34]. Cellular lipids may simply provide more energy reserves for stress responses. Alternatively, a UV-induced morphological variation maybe better adapted to adverse condition/lipid production. Since UV causes genetic mutations [36, 37], UV may have acted as a selection pressure during algal evolution [38, 39]. At high UV-C doses, the release of lipid bodies became apparent as cell structures disintegrated (Fig. 1). Considering the difficulties often experienced for lipid extraction due to rigid cellular structures, UV-C-implemented cell damage may also contribute to higher lipid extraction efficiencies.

Compared to previous lipid induction techniques [9, 40, 41], UV-C radiation resulted in faster lipid biosynthesis stimulation within 24 h (Fig. 2-4). Depending on the quantification method used, the total lipid or TAG production approximately doubled compared to lipid induction by nutrient depletion only. Under outdoor conditions, TAGs were induced by lesser extend (Fig. 5b), perhaps because controls also received UV-containing solar irradiation or because biomass was harvested after the peak in lipid fluorescence of Nile red-stained cells (Fig. 4a). This emphasizes that further fine-tuning of UV-C exposure and harvesting times may be required to maximize TAG induction while minimizing cell mortality, and this is likely to vary for different microalgae, cultivation systems, and climatic conditions.

Although the total lipid content increased by UV-C radiation to different extents, an alteration of fatty acid profiles only occurred at low doses (100 and 250 mJ/cm^2 for laboratory- and 48 J/cm^2 for outdoor-grown cultures). Interestingly, the decrease of C18 and C20 SFAs corresponded to the increase of C16:2, C16:4, C18:1, C18:2, C18:3 and C20:4 USFAs (Fig. 5; Supplementary Fig. 2, 3 and 9). A similar result was obtained in UV-B-treated *Spirulina*, concomitant with deleterious effects on thylakoid membrane integrity and protein profiles [42]. Amongst USFAs, the increment of PUFAs was the main change in the present study. As PUFAs are involved in cell repair and growth [43], it is conceivable that *Tetraselmis* cells attempted to repair the photo-damage caused by UV-C. However, photo-damage appeared irreversible at high UV-C doses since the lipid profile did not change in 24 h after 500 or 1000 mJ/cm^2 exposure.

The fatty acid synthesis pathway of *Tetraselmis* sp. is regarded to be similar to that of *Chlamydomonas reinhardtii* [44] where fatty acid desaturation results in insertion of double bonds into pre-formed fatty acid chains. Therefore, we speculate that the increase of C16:2, C16:4, C18:1, C18:2, C18:3 and C20:5 unsaturated fatty acids were actually the products of the desaturation of C16, C18 and C20 SFAs in *Tetraselmis* sp. M8 (Supplementary Fig. 9). Low UV-C dosage may help to convert SFAs to unsaturated fatty acids with a significant increase in PUFAs. In microalgae, the deleterious effect of UV light on thylakoid membrane integrity leads to the production of reactive oxygen species (ROS) [42] (Supplementary Fig. 4). PUFAs have a strong affinity or absorption to ROS [45], therefore, the increment of PUFAs can be interpreted as a defence response against UV-C-generated cellular ROS.

The increase in USFA when compared to SFA in open raceway ponds confirmed the results obtained for lab-scale studies. This may have implications to biodiesel fuel properties that may possess better cold flow properties but maybe more prone to oxidation. Further studies should be carried out to determine whether biosynthesis of nutraceuticals such as eicosapentaenoic acid (EPA) can be manipulated by UV-C radiation. A significant two-fold increase of EPA was detected at laboratory-scale (Supplementary Fig. 3). UV-C treatment also significantly reduced co-cultured microorganisms (Supplementary Fig. 8), which may be advantageous for water recycling and for minimizing contamination during continuous cultivation, an important factor for commercial production. Figure 4a suggests that some lipids are consumed by microalgae after peak accumulation and during harvesting (with or without UV-C), but final UV-C treatment for settling may reduce lipid degradation as cell mortality increases.

In summary, this study used low exposure of UV-C radiation to induce lipids and harvest microalgae at the same time. Outdoor cultivation of *Tetraselmis* sp. M8 in open raceway modules showed that UV-C radiation could be a rapid and effective process for LIS of flagellate microalgae. UV-C radiation could also control co-cultured microorganisms, facilitate water recycling and may assist in subsequent lipid extraction by pretreating cells, all of which could potentially be well integrated in a microalgae biorefinery concept. UV-C-mediated LIS may provide an important step towards commercial microalgal biofuel production.

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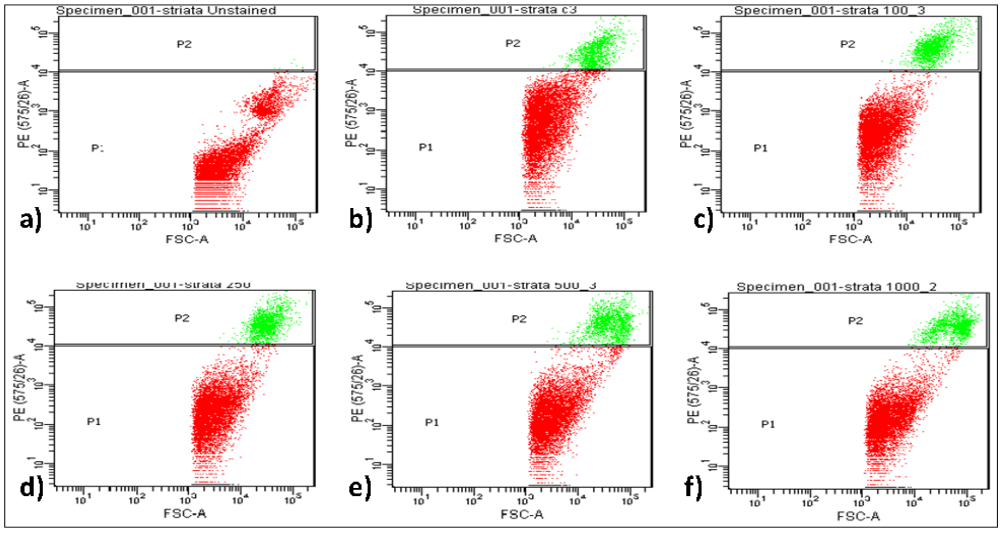
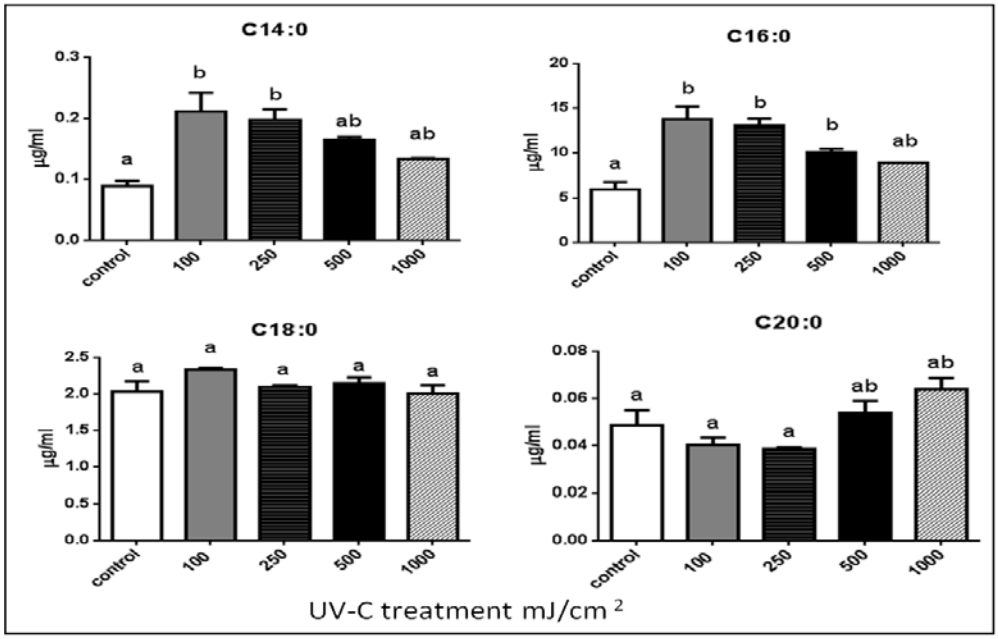
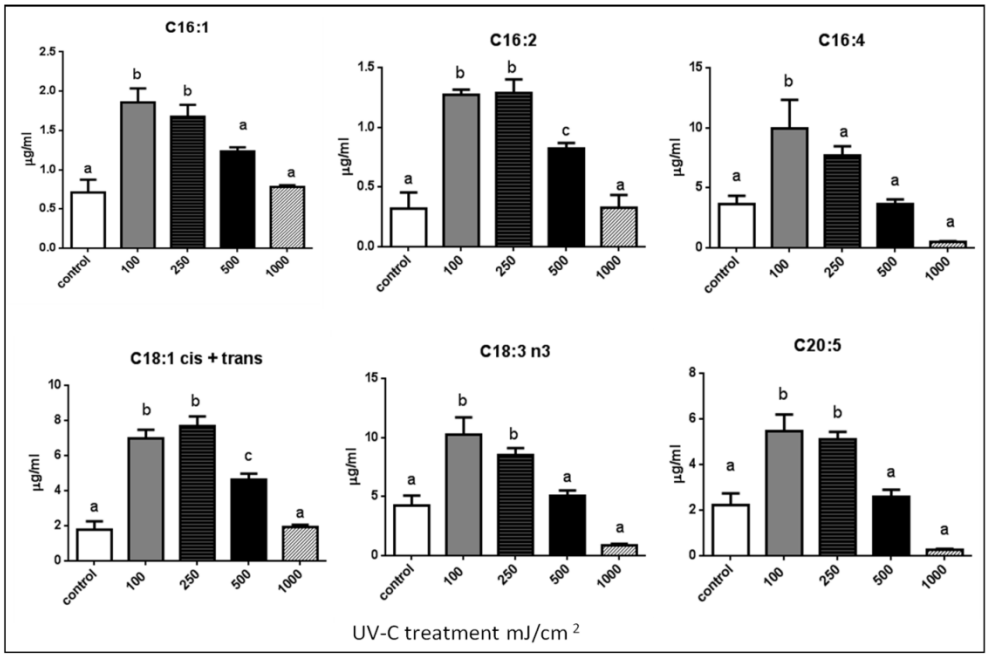


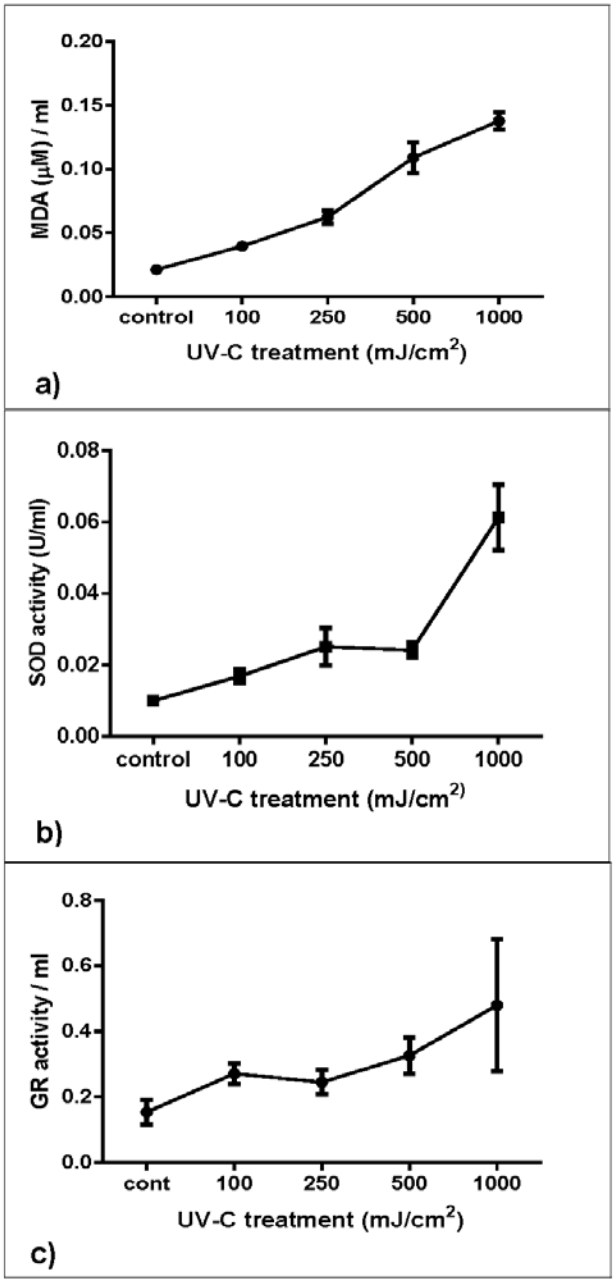
Supplementary Figure 1. Fluorescence-Activated Cell Sorting (FACS) analysis of *Tetraselmis* sp. M8 cells that received different UV-C dosages. Shown are unstained Nile red control (**a**), untreated control stained with Nile red (**b**) and UV-C treated populations at 100 mJ/cm^2, 250 mJ/cm^2, 500 mJ/cm^2 and 1000 mJ/cm^2 (**c-f**; respectively). Signals from unstained control cells were used as a baseline cut-off for the P1 population (red) and cells above this cut-off were marked the as lipid-producing P2 population (green). The Y-axes show fluorescence intensities at a PE excitation wavelength of 575 nm and the X-axes show the forward scatter based on cell size.



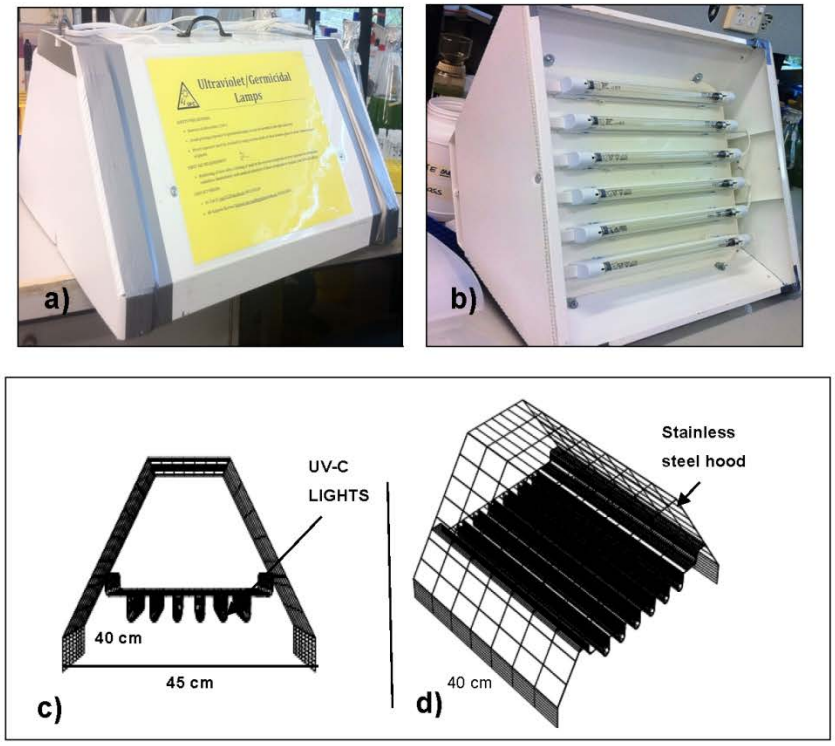
Supplementary Figure 2. Comparison of different saturated fatty acids present in *Tetraselmis* sp. M8 microalgae following different dosages of UV-C radiation. Shown are mean values ± SEs from three separately-grown and -treated cultures. Different letters show statistically significant differences ($P<0.05$).



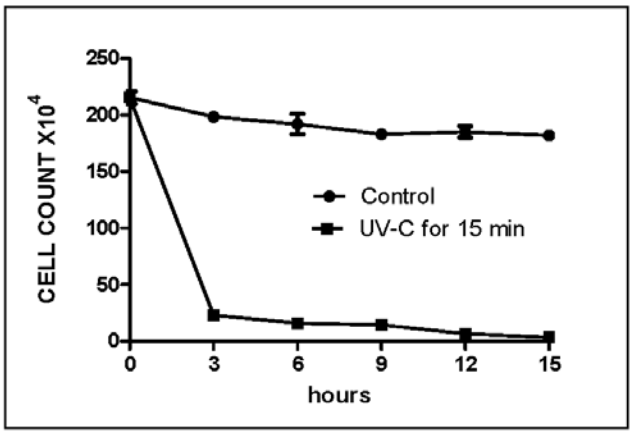
Supplementary Figure 3. Comparison of different unsaturated fatty acids present in *Tetraselmis* sp. M8 microalgae following different dosages of UV-C radiation. Shown are mean values ± SEs from three separately-grown and -treated cultures. Different letters show statistically significant differences (P<0.05).



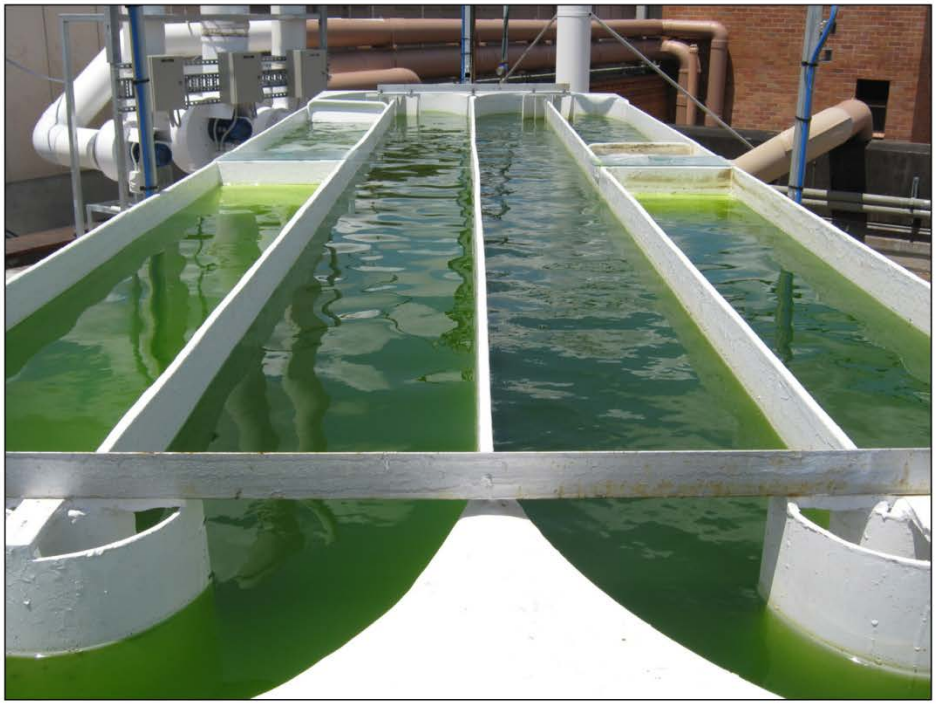
Supplementary Figure 4. Enzymatic assays of UV-C-stressed and mock-treated *Tetraselmis* sp. M8 cells at different dosages. a) Decomposition of unstable peroxides (TBRAS) assay, **b**) Superoxide dismutase (SOD) assay, **c**) Glutathione reductase (GR) assay (normalized to cell count). Values are mean ± SEs from three separately-grown and -treated cultures.



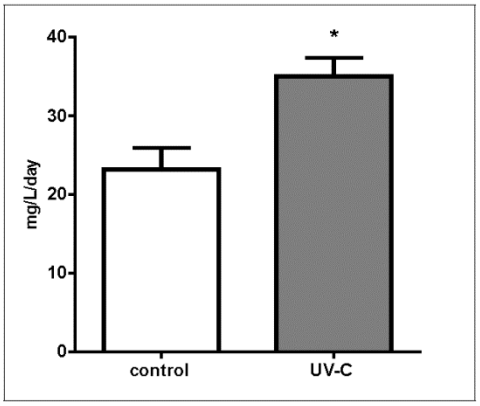
Supplementary Figure 5. Equipment for small-scale outdoor microalgae UV-C treatment. Shown are the design of the UV-C chamber (**a-d**) and the experimental setup of the UV-C treatment for microalgae in 20 L cultures (**e**). The UV-C chamber delivered approximately 12 J cm^{-2} h^{-1}.



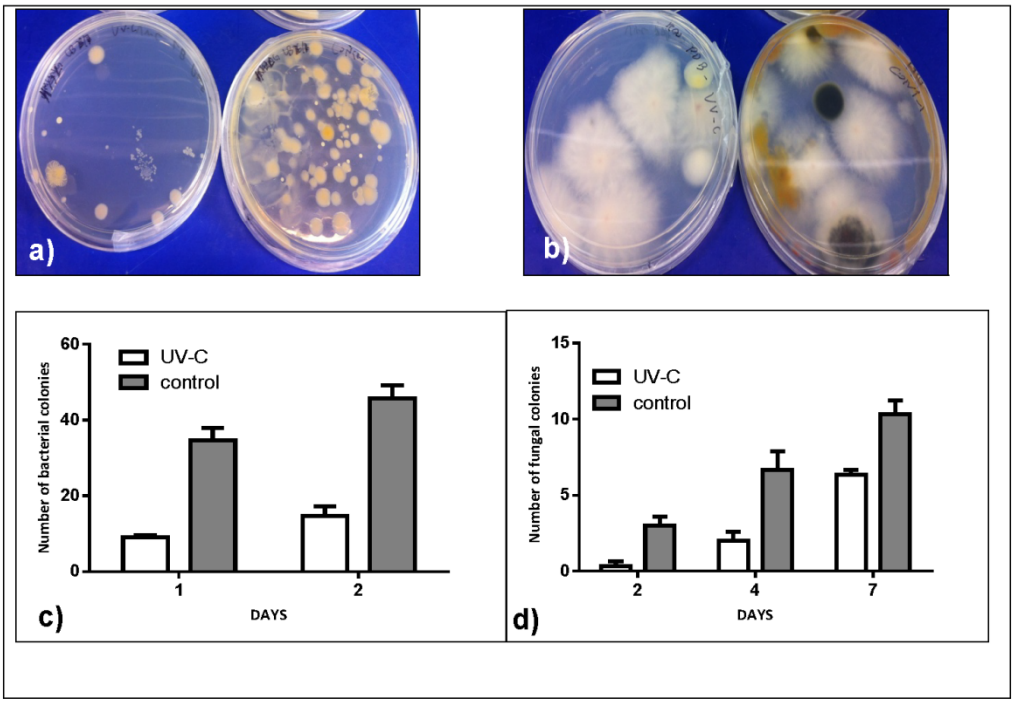
Supplementary Figure 6. Timecourse analysis of *Tetraselmis* sp. M8 culture settling following UV-C treatment. Shown are cell counts in the medium from three independent 20 L outdoor cultures with a depth of 20 cm and UV-C exposure of 3 J/cm^2.



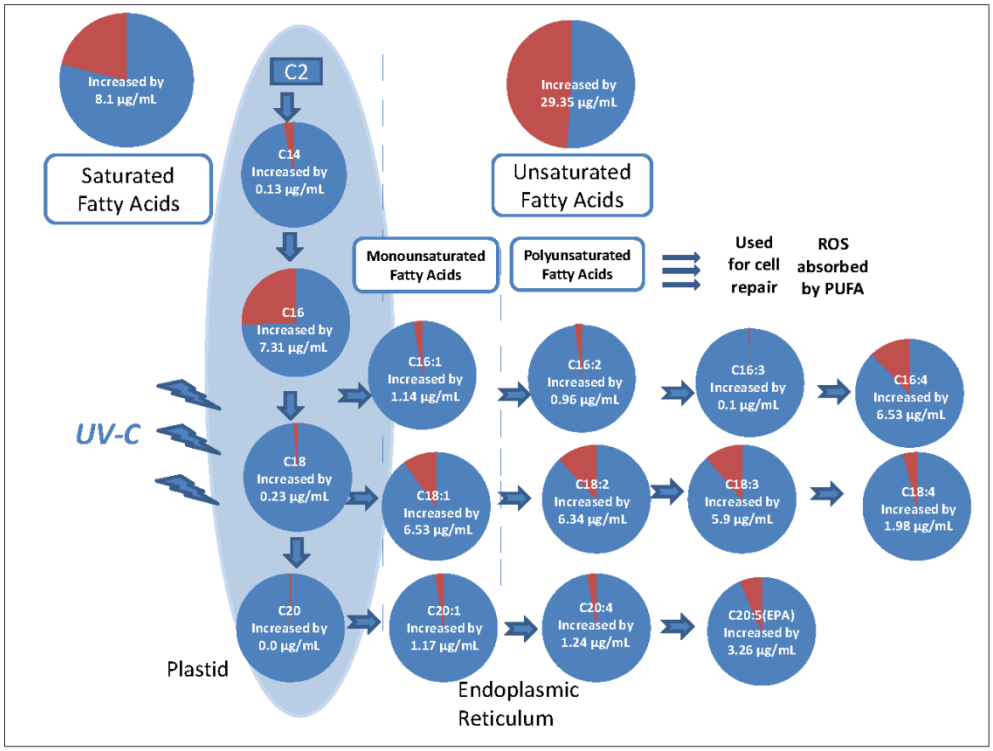
Supplementary Figure 7. Photograph of two identically designed 1000 L-raceway ponds whose cultures were aerated and circulated by airlifts with diagonally-applied pressured air.



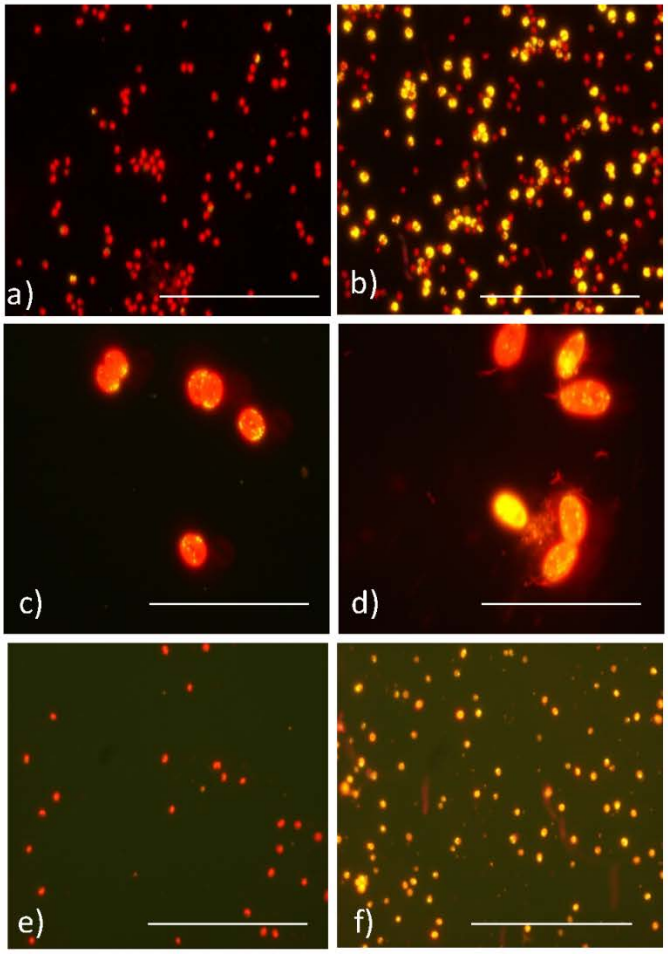
Supplementary Figure 8: Lipid productivity of *Tetraselmis* sp. M8 culture following UV-C treatment. Bars with (*) show significant differences ($P<0.05$). Shown are mean values ± SEs from three separately-grown and -treated outdoor raceway cultures.



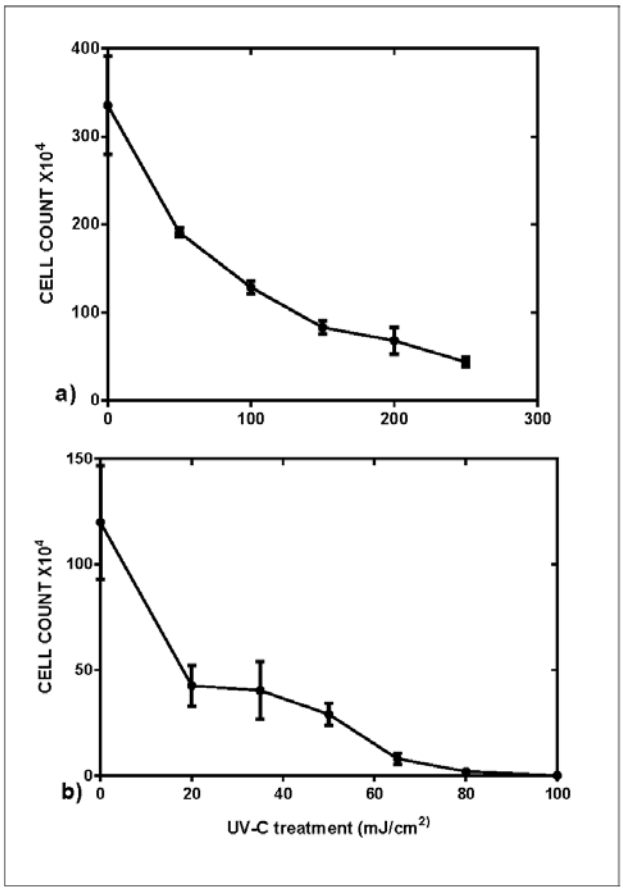
Supplementary Figure 9. UV-C treatment reduces microbial presence in algal cultures. a,b) LB and PDB plates, respectively, with UV-C-treated used algae culturing medium (left plate) showing significantly fewer bacterial and fungal colonies, respectively, when compared to the mock-treated control (right plate). **c,d**) Quantification of culturable bacterial (c) and fungal (d) colonies shown as mean values with SEs from three separately-grown and -treated raceway algal cultures.



Supplementary Figure 10. Effect of low dosage of UV-C radiation on the fatty acid synthesis pathway. UV-C radiation might help in conversion of saturated fatty acids that serve as a storage function in plastids to unsaturated fatty acids that may reduce oxidative cell damage. Shown are total and percentage increases in pie charts for each fatty acid.



Supplementary Figure 11. Increase in lipid fluorescence (yellow) before and after UV-C radiation in different microalgae. **a,b)** Control and UV-C treated (100 mJ) *Chlorella* sp. BR2; **c,d)** Control and UV-C treated (100 mJ) *Tetraselmis chui;* **e,f)** Control and UV-C treated (250 mJ) *Nannochloropsis* sp. BR2. Photographs were taken of Nile red-stained microalgae at 40x magnification (bar=50 µm) at 24 h after treatment.



Supplementary Figure 12. Induced settling of microalgae at different dosages of UV-C radiation at 6 hours after treatment: a) *Dunaniella salina;* b) *Tetraselmis chui*. Shown are mean values with SEs from three separately-grown and -treated 50 mL algal cultures.

Chapter 5: Rapid induction of omega-3 fatty acids (EPA) in Nannochloropsis sp. by UV-C radiation (Submitted)

Overview

Experiments using UV-C radiation on C*hlorella* sp. BR2 *and Tetraselmis* sp. M8 showed that this external stress can stimulate lipid accumulation, especially PUFAs. Hence, to test weather UV-C radiation could be used to induce high value fatty acids in *Nannochloropsis* sp. BR2. In particular the production of eicosacenoid pentanoic acid (EPA), an important omega-3 fatty acids with proven health benefits, was targeted. Combined sequential stress treatments were carried out to achieve this aim.

Key Findings

- UV-C treatment of *Nannochloropsis* sp. resulted in higher total polyunsaturated fatty acids contents, with up to 40% EPA of total fatty acids.
- The treatment also led to a significant increase of volumetric omega-3 productivity.
- A combined sequential approach, using UV-C and nutrient starvation may provide a promising approach to produce large amounts of algal omega-3, in an effort to reduce dependency on global fish stocks.

Chapter 5: Rapid induction of omega-3 fatty acids (EPA) in *Nannochloropsis* sp. by UV-C radiation (Submitted)

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Abstract

Omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) provide substantial health benefits. As global fish stocks are declining and in some cases are contaminated with heavy metals, there is a need to find a more sustainable land-based source of these essential fatty acids. The oleaginous microalga *Nannochloropsis* sp. has been identified as a highly efficient producer of omega-3 fatty acids. In this study, we present a new process to rapidly induce biosynthesis of essential fatty acids, including EPA in *Nannochloropsis* sp. BR2. Short exposure to UV-C at a dose of 100 or 250 mJ/cm2 led to a significant increase in total cellular lipid contents when compared to mock-treated controls. A low dosage of 100 mJ/cm^2 also led to a two-fold increase in total EPA content within 24 h that constituted 30% of total fatty acids and up to 12% of total dry weight at higher dosages. UV-C radiation may find uses as an easily applicable external inducer for large-scale production of omega-3 production from microalgae.

Introduction

Within the last decade microalgae have emerged as a potential source of renewable energy and nutraceutical products. Of particular interest are long chain polyunsaturated fatty acids (LC-PUFAs), including omega 3 and 6 (ω-3,6) fatty acids. Omega-3 and 6 fatty acids are essential components for the growth of higher eukaryotes (Ward and Singh,

2005; Adarme-Vega et al. 2012;). Nutritionally, arachidonic acid (AA, 20:4, ω-6), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) are the most important fatty acids belonging to this group of bioactive compounds. AA and EPA are components of mammalian cell membranes and are also precursors of the eicosannids, including the prostaglandins, a family of biological effectors involved in inflammatory responses, blood pressure regulation, blood clotting and cell signaling (Kinsella et al. 1990). These compounds have been proven to help in neonatal retinal and brain development, as well as cardiovascular health and disease prevention (Carlson et al. 1993; Crawford 2000; Gill and Valivety 1997; Neuringer et al. 1988).

Current sources of ω-3,6 LC-PUFAs, EPA and DHA are marine fish oil (e.g. from mullet and krill), however global fish stocks have been in decline since the late 1980s (Worm et al. 2006). Moreover, traces of heavy metals have been found in marine fish, rendering these fish harmful to consumers (Bourdon et al. 2010). Fish oil is also not suitable for vegetarians and the odor makes it unattractive. Microalgae are the primary producers of these compounds. Their ability to grow under autotrophic, mixotrophic and heterotrophic culture condition and on non-arable land with limited water recourses makes them a preferred choice for commercial production of ω-3,6 PUFAs, EPA and DHA. There is a wide range of lipid induction techniques in microalgae, such as the use of nutrient stress, including nitrogen and/or phosphorus starvation, light irradiation, pH or temperature change, and exposure to heavy metals or other chemicals (Sharma et al. 2012). Of all the microalgae studied, *Nannochloropsis* sp. are known to produce high amounts of ω-3,6 LC-PUFAs, EPA [10-13]. For example, *Nannochloropsis oculata* cultivated under nitrogen limitation conditions and an increase in temperature from 20°C to25°C resulted in an increase in total lipids by two fold increase (Converti et al. 2009). Moderated use of UV-A radiation for seven days could lead to an increased production of fatty acids in *Nannochloropsis* sp. (Forján et al. 2011) and in a study carried out by Srinivas and Ochs (2012) on *N. oculata* the effect of UV-A at different levels resulted in significantly increased lipid to chlorophyll ratio.

Although these protocols highlighted the possibility to increase lipid production based on laboratory research, there are still many concerns for their application, such as difficulties with culture maintenance, elongated cultivation periods/low lipid productivity, high cost for large-scale cultivation, oil extractability and potential environmental impacts. In most

cases, it is also uncertain whether the induced lipid production during cultivation was derived from the sacrifice of algal growth or not. Moreover, under nutrient stress condition in *Nannochloropsis* sp. usually higher accumulation of SFA is favored when compared to LC-PUFA's.Therefore, there is a desire to develop an effective protocol to stimulate lipid production more importantly LC-PUFA, while sustaining cell growth. Previous published study on *Tetraselmis* sp (M8) indicated that exposure of M8 cells to small dosage of UV-C 100-250 mJ/cm^2 radiation resulted increase LC-PUFA, especially C18:4, followed by twofold increase of ω-3 fatty acids, However the actual production of ω-3 fatty acids was only 8 µg/ml. The study also highlighted the impact to low dosage of UV-C radiation on fatty acid biosynthesis pathway in converting saturated fatty acids (SFA's) into LC-PUFA's. Based on these results it was hypothesized that the contents of these LC-PUFA can be further increased in *Nannochloropsis sp by low dosage of* UV-C radiation *as* naturally these species is known to produce high amounts of LC-PUFA's especially EPA.

Materials and methods

Laboratory-scale microalgae culturing and UV-C treatment

Nannochloropsis sp. BR2*,* family (Eustigmataceae) was originally collected from the Brisbane River and was cultured in the Algae Biotechnology Laboratory at The University of Queensland (Lim et al. 2012). Cultures were grown in f/2 medium (Guillard and Ryther 1962) in artificial sea water obtained from CSIRO at 23°C on an orbital shaker (Thermoline) at 100 rpm with 12 hours of light and dark rhythm. When the cultures were at late exponential growth phase and the cell densities reached 7.5x10^5 cells/mL, cultures were subjected to UV-C radiation. The method for UV-C treatments has previously been reported (Sharma et al. 2014).

Lipid fluorescence analysis and GC-MS analyses

Nile red staining was conducted) followed by flow cytometry analysis using a BD LSR II: Analyzing flow cytometer with 573 nm of excitation wavelength as described previously (Sharma et al., 2014). A total of 10,000 cells were counted in each sample. A gate was set to separate lipid producing fluorescence-activated cells and inactivated cells based on the analysis of cells without Nile red staining. The background absorbance from chloroplast auto-fluorescence was subtracted from each sample using mock-treated (control)

Nannochloropsis sp. BR2 cultures that were not Nile red stained. Qualitative observation was conducted simultaneously by fluorescence microscopy as described below. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out as described previously (Sharma et al., 2014).

Dry weight measurements

A total of 5 mL of the culture were used for dry weight measurements. The culture was filtered through a 0.27 µm glass fiber filter (Millipore) which was pre-weighed and pre-washed with 1 mL distilled water in a vacuum-subjected filter unit (three biological replicates were used from each culture (UV-C treated and mock-treated control)). After filtration, the filters were kept in individual Petri dishes to avoid contamination and dried in a drying oven for 24 h at 80°C with the plate lid half open, prior to weighing.
To determine the dry weight the following formula was used:

$$\text{Dry weight mg/L} = \frac{\text{Filter dry weight - Filter pre-weight}}{\text{Filtered volume mL} \times 1000}$$

Dry weight in mg/L was determined from the average of three weight measurements for each replicate.

Analytical methods

Data for growth rates and lipid productivities were statistically analyzed by one-way analysis of variance (ANOVA) with different microalgal cultures as the source of variance and growth rate or lipid productivity as dependent variables. This was followed by Turkey's multiple comparisons test ($P>0.05$) where appropriate.

Results

Low UV-C exposure leads to increased lipid accumulation in *Nannochloropsis* cells

Nannochloropsis sp. cells have previously been shown to accumulate significant amounts of lipids after nutrient deprivation and were identified as one of the most promising autotrophic microalgal sources for omega-3 fatty acid production (Huerlimann et al., 2010; Lim et al. 2012). Preliminary experiments using combined stresses of nutrient starvation and UV-C radiation on *Tetraselmis* sp. have indicated that UV-C exposure can be an

efficient stimulus to induce LC-PUFA production, including EPA (Sharma et al. 2014). Thus in an effort to optimise EPA production in *Nannochloropsis*, UV-C treatment was applied to nutrient-starved *Nannochloropsis sp. BR2 cells*. Following Nile red staining, it became quickly apparent that lipid fluorescence intensities (yellow florescence) became stronger with an increase of UV-C radiation from 100 to 500 mJ/cm^2 (Fig. 1a). The reported cell rupturing that was observed in *Tetraselmis* sp. at 1000 mJ/cm^2 (Sharma et al., 2014) was not apparent in *Nannochloropsis* sp. BR2, suggesting that cell walls are quite rigid (Fig 1a). Based on the presence of green color in live cells and grey or brown color in dead cells, survival rates were determined by cell counting in a hemocytometer. This showed a gradual decline of live cells when UV-C radiation increased (Fig. 1b). The cell survival rates reached half (LD50) at UV-C radiations of 100 - 250 mJ/cm^2, and at 500 mJ/cm^2 and 1000 mJ/cm^2 only 5% of the cells had survived. There was no significant difference observed in the cell size at different UV-C radiation intensities as had previously been observed for *Tetraselmis* sp. (Sharma et al. 2014).

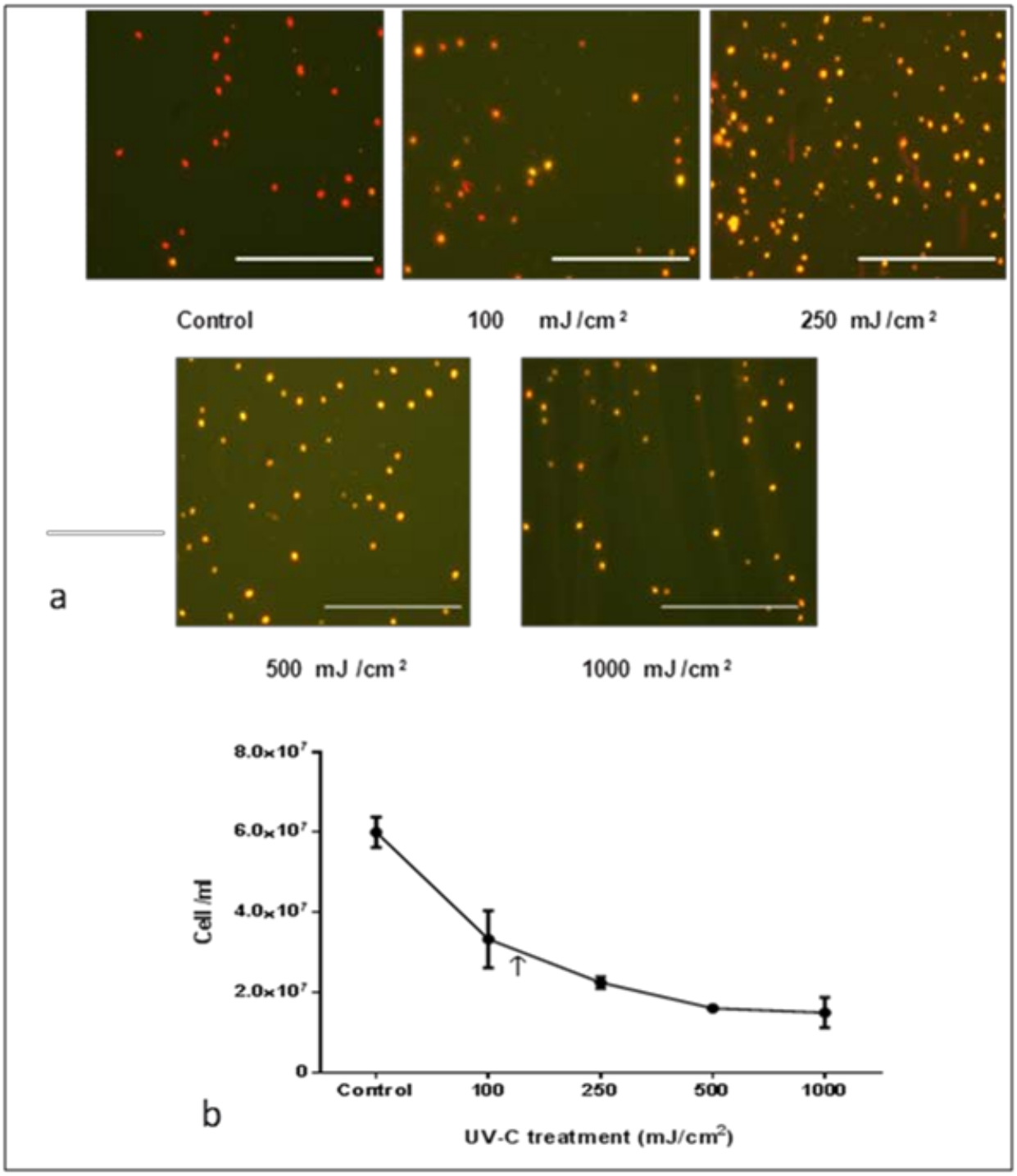


Figure 1: Nile red-stained cells of *Nannochloropsis* sp. BR2 that received different doses of UV-C exposure after nutrient starvation. a) Cells with maximum lipid fluorescence (yellow) can be observed at 250 mJ and 500 mJ UV-C radiation. Cells are shown at 40x magnification (bar=50 µm) at 24 h after treatment. b) Kill curve of *Nannochloropsis* sp. BR2, showing the number of cells that survived UV-C treatment at different dosages. Shown are mean values ± SEs from three independent treatments. The arrow indicates the LD50 value.

With an increase of UV-C radiation, the algal cells were divided into two different populations of P1 and P2, according to the fluorescence intensity (Fig 2 a-f). P1 population presented the auto-fluorescence of chloroplasts in the cells, corresponding to control cultures which were not stained by Nile red, while P2 represents Nile-red fluorescing cells (Fig 2a). In nutrient-starved control cultures, about 75% and 35% of the cells were present in P1 and P2 populations, respectively. Whereas, as the UV-C radiation increased from 100 to 1000 mJ/cm^2 a sharp decline was observed in the P1 population which was accompanied by sharp increase in the P2 population (Fig 2 a-f). P1 started to decline sharply with an increase of UV-C radiation. The P2 proportion increased significantly from 100 to 500 mJ/cm^2 (P<0.05) and was maintained at 95% between 500 and 1000 mJ/cm^2 (P>0.05) (Fig 3a). Overall an increase in Nile red-fluorescing P2 population and a decrease in unstained P1 population with an increase in UV-C intensity was observed that were consistent with results previously obtained for *Tetraselmis* sp. (Sharma et al., 2014).

Based on the average fluorescence value, P1 cells displayed much less fluorescence intensity than P2 (Fig 3b). Different from P1, P2 cells had a big variation of fluorescence intensity that correlated to the total lipid intensity (Fig 3b). Compared to the mock-treated control cells, the lipid fluorescence intensity of algal cells was significantly increased with the increase in UV-C radiation. However, there was no significant difference between the control (0 mJ/cm^2) and 100 mJ/cm^2 –treated cells (Fig 3c). As UV-C dosage was increased to 250 mJ/cm ^{2} and 500 mJ/cm^2, the total lipid fluorescence in both the cultures was nearly increased by two fold when compared to the control (Fig 3c). Subsequently, the lipid intensity of P2 cells sharply dropped to the control level at 1000 mJ/cm^2 (Fig. 3b,c). However, the total lipid fluorescence was still increased significantly at 1000mJ/cm^2 when compared to the mock-treated control (Fig 3c).

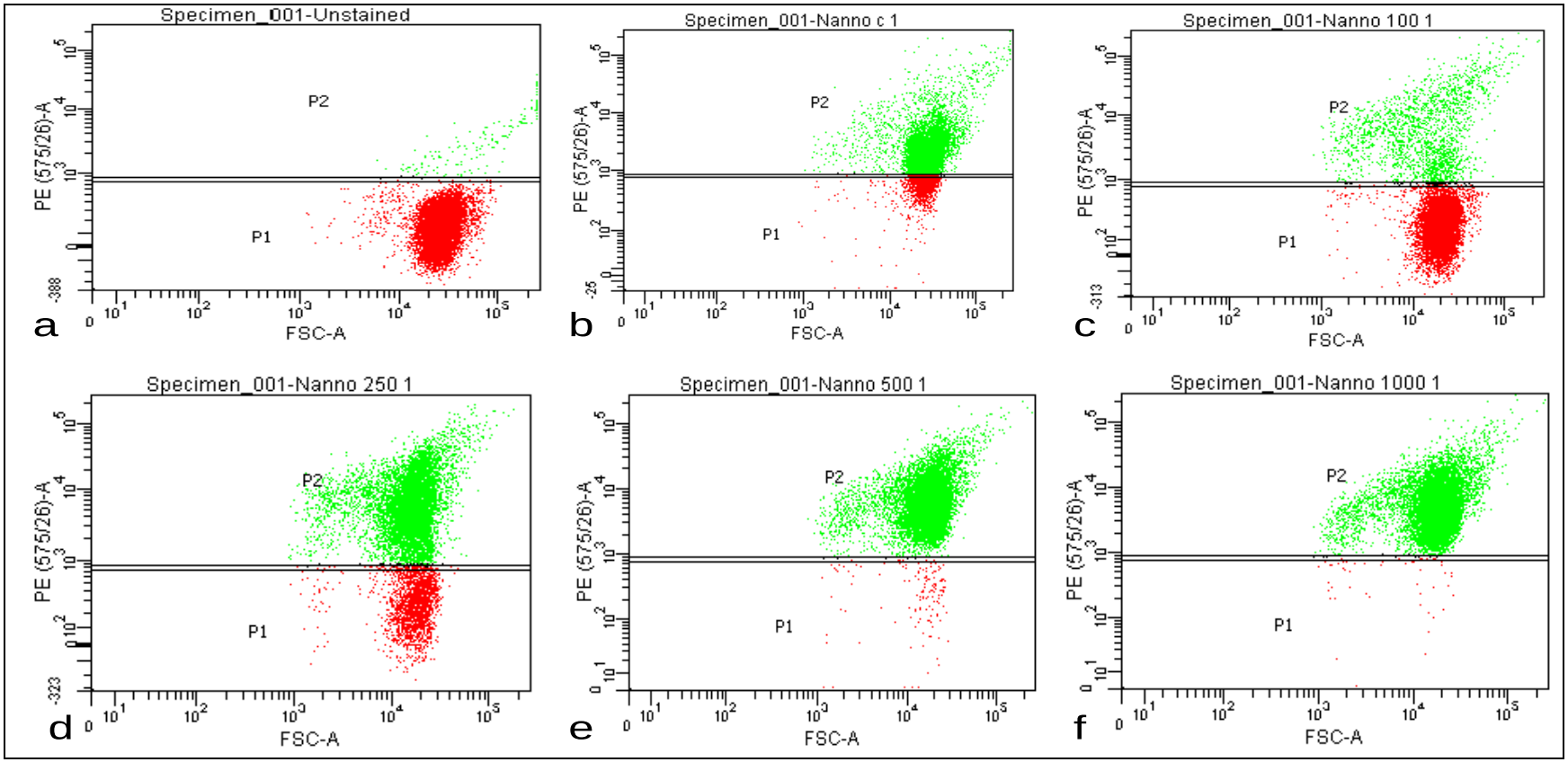


Figure 2: FACS analysis of *Nannochloropsis* sp. BR2 unstained cells (a), Nile red-stained mock-treated control cells (b), UV-C treated cells (c-f) at 100, 250, 500 and 1000 mJ/cm^2, respectively, showing P1 (background fluorescence) and P2 (Nile red fluorescence) populations . The y-axis shows fluorescence intensity at phycoerythrin excitation wavelength of 575 nm and the X-axis shows the forward scatter based on cell size.

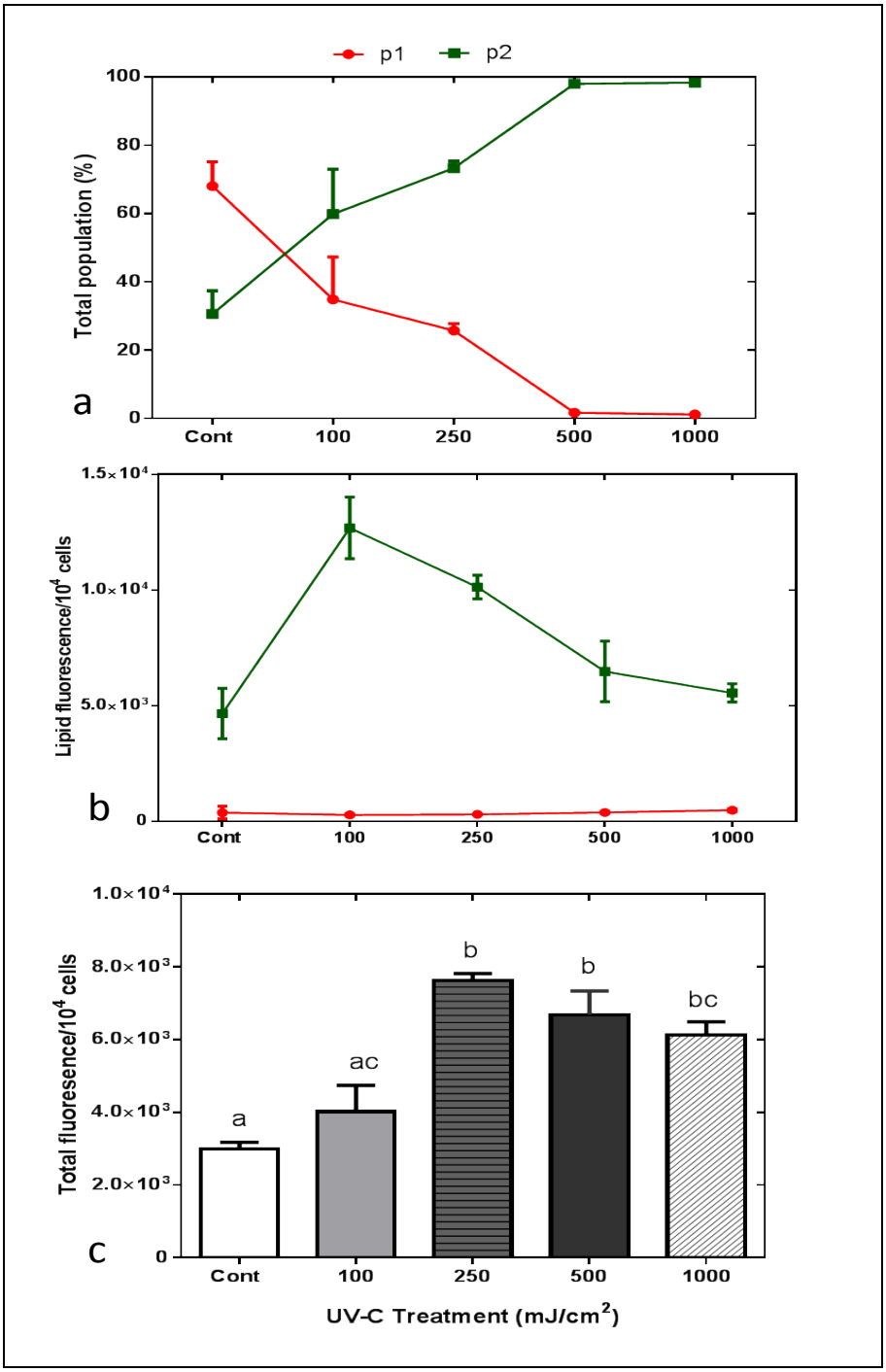


Figure 3: Lipid induction in *Nannochloropsis* sp. BR2 at 24 h after receiving different UV-C dosages. FACS analysis of Nile red-stained cells showing distribution (a) and lipid fluorescence (b) of low (P1) and high (P2) fluorescence cell populations and of the total population (c). Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with different letters indicate significant differences (P<0.05).

Fatty acid profiling in *Nannochloropsis* following UV-C radiation shows lipid induction and a shift towards LC-PUFAs

To further quantify the ability of UV-C stress to increase cellular fatty acid contents and to profile their composition, GC-MS analyses were carried out. These confirmed the results obtained from flow cytometry, showing a significant total fatty acid increase (*P*=0.002, *P*=0.031; respectively) of UV-C-treated cultures (100 and 250 mJ/cm^2) compared to mock-treated nutrient-starved control cultures, whereas cultures treated with 500 and 1000 mJ/cm^2 showed no significant difference (Fig. 4a). Moreover, in cultures treated with 100 and 250 mJ/cm^2, the amount of unsaturated fatty acids (USFA) significantly increased and also the proportion of USFA compared to total fatty acids (Fig. 4a,c). Amongst the saturated fatty acids (SFA), the highest increase was observed for C16 (palmitic acid), followed by C14 (Fig. 4b). On the other hand, C18 and C20 did not show any increase in cultures treated with 100 and 250 mJ/cm^2 UV-C, whereas in cultures treated with 500 and 1000 mJ/cm^2 there was no significant increase of any fatty acid (Supplementary Fig. 1 and 2). When comparing different USFA, cultures treated with 100 and 250 mJ/cm^2 UV-C showed significant increases for all detected USFA, most notably C20:5 (EPA) which accounted to be nearly 30% of the total fatty acid, and was increased highly significantly at 100 mJ/cm^2 (*P*=0.0075) followed by 250 mJ/cm^2 (*P*= 0.0308) (Fig. 4c). Other MUFAs namely C16:1, C18:1 cis+trans, and PUFAs C20:4 were also significantly increased, (Supplementary Fig. 2). EPA contents in cellular dry weight was measured to determine suitability of *Nannochloropsis* for large-scale EPA production. As shown in Fig. 5, EPA constituted up to 12% of total dry weight at higher dosages, however a lower dose of UV-C resulting in a lower EPA content maybe preferred to ensure high overall EPA productivity.

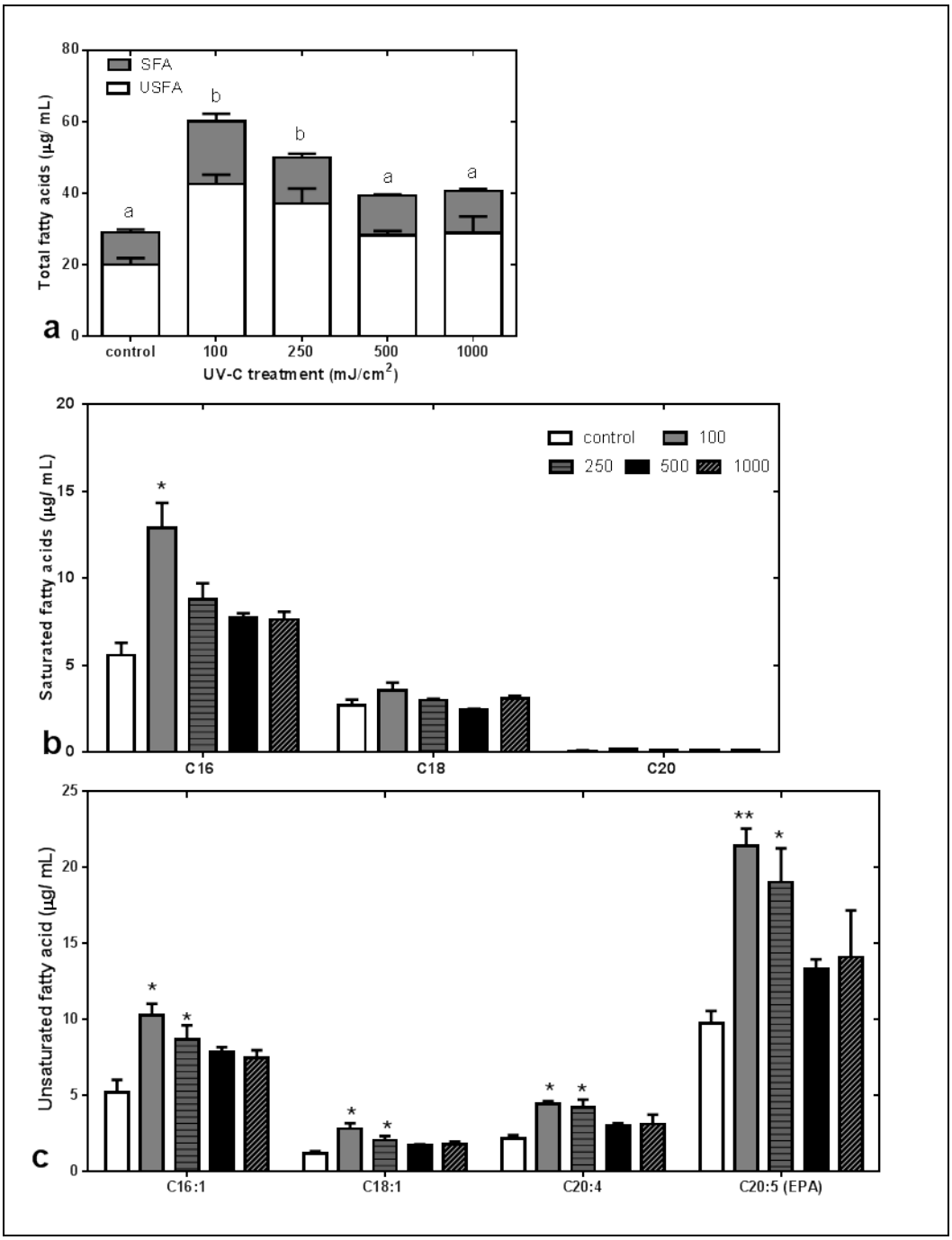


Figure 4: Fatty acid profiling and quantification by GC-MS showing (a-c) total fatty acids as well as saturated (SFA) and unsaturated (USFA) fatty acids, respectively, produced by different UV-C-treated *Nannochloropsis* sp. BR2 cultures. Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with (*) indicate

significant differences (P<0.05) and bars with (**) indicate highly significant difference (P<0.01).

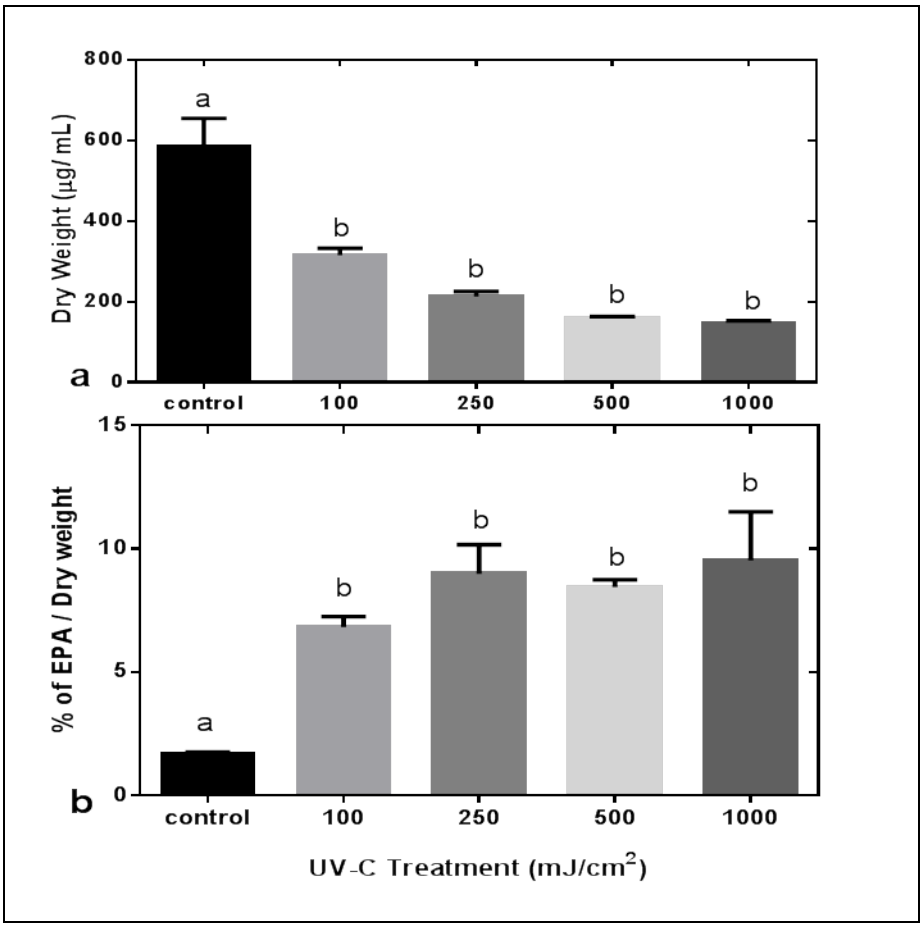


Figure 5: Dry weight and EPA proportion changes in *Nannochloropsis* sp. BR2, following nutrient starvation and different UV-C exposures. Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with different letters indicate significant differences (P<0.05).

Discussion

Consistent with the previous study conducted on *Tetraselmis* sp. by Sharma et al. (2014), it was reported that the low dosage of UV-C radiation could also stimulate lipid biosynthesis in *Nannochloropsis* sp. and induce the production of PUFAs. A radiation of

100 and 250 mJ/cm^2 was optimal to induce total lipids content especially USFAs which were increased by two fold followed by SFA's (Fig. 4).

A notable difference when comparing flow cytometry and GC-MC data was observed. The total fluorescence shown by flow cytometry analysis was significantly highest at 250 and 500 mJ/cm^2 followed by 1000 mJ/cm^2. Whereas, GC-MS analysis indicated maximum total fatty acids at UV-C exposure of 100 and 250 mJ/cm^2. This difference in the lipids fluorescence can be attributed to the P2 population as the percentage of this population was higher in the culture treated at 250 mJ/cm^2 (75%) and 500 -1000 mJ/cm^2 (95%). Thus it was understood that the high proportion of P2 population and the considerably high number of live cells resulted in high total lipid fluorescence. But when considering the fluorescence of individual P2 cells, the culture treated at 100 mJ/cm^2 displayed the highest fluorescence followed by 250 mJ/cm^2 and 500 mJ/cm^2 which corresponds well to the results obtained by GC-MS. Moreover, when considering the dry weight measurement, as expected there was a linear decline in the total dry weight due to increased cell death at higher dosages (Fig. 5). But on the other hand, there was a significant increase in the percentage of cellular EPA with maximum values at 1000 mJ/cm^2 (12% of total dry weight; Fig 5).

Cellular lipids may simply provide more energy reserves for stress responses. Alternatively, a UV-induced morphological variation maybe better adapted to adverse condition/lipid production. Since, UV-C causes genetic mutations (Guihéneuf et al. 2010; Rothschild 1999), UV may have acted as a selection pressure during algal evolution (Cockell 2000; Cockell and Raven 2007). In the previous study conducted on *Tetraselmis* sp. by Sharma et al. (2014) it was reported that at the higher dosage of UV-C (1000 mJ/cm^2) the release of lipid bodies became apparent as cell structures disintegrated, However cell rupturing was not observed in this study, the possible reason for this would be the relatively small cell size of the *Nannochloropsis* cells when compared to *Tetraselmis* sp. cells, also *Nannochloropsis* sp. cells reportedly possess rigid cell walls when compared to other microalgal species (Iqbal and Theegala 2013).

When comparing to other lipids induction techniques (Sharma et al. 2012; Takagi et al. 2006; Wang et al. 2008), UV-C radiation resulted in faster lipid biosynthesis stimulation within 24 h (Fig. 1-4). Of all the fatty acids measured, a significant increase was observed in the production of the omega-3 fatty acid EPA which was induced by 2.5 fold and accounted to be approx. 30% of the total fatty acids (Fig 4c) followed by a two fold increase in C18:1, C18:4, C20:4 (Supplementary Fig. 2). The previous study reported that UV-C radiation on *Tetraselmis* sp. lead to an increase in reactive oxygen species (ROS) production (Sharma et al., 2014). PUFAs have a strong affinity or absorption to ROS (Bouhamidi et al. 1998), therefore, the increment of PUFAs in this study can be interpreted as a defence response against UV-C-generated cellular ROS.

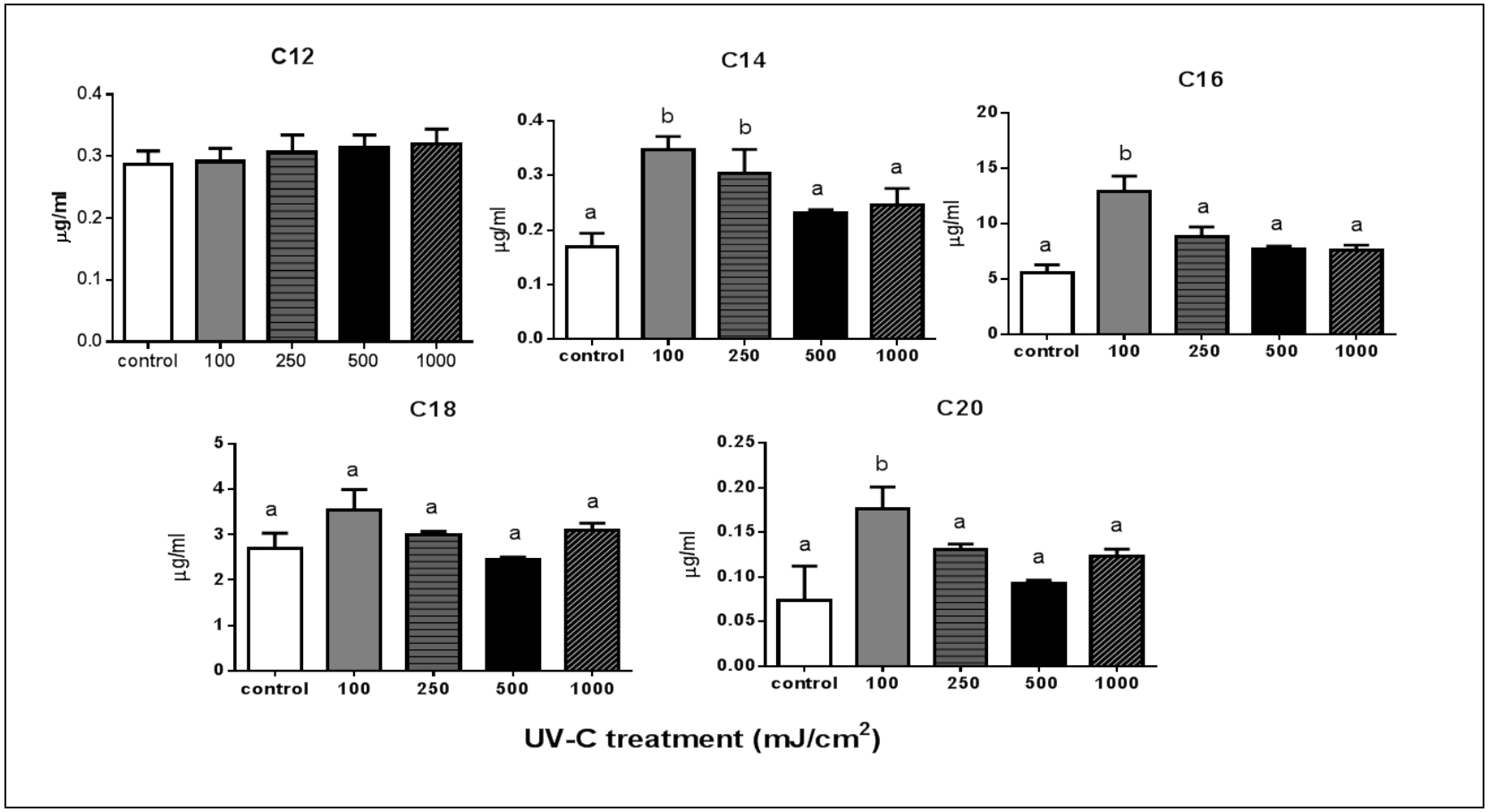
The role of PUFAs as an antioxidant and an agent to prevent cell damage has been well documented in many studies on plants and animals (Bartsch et al. 1999; Cheeseman and Slater 1993; Lenzi et al. 2000; Machlin and Bendich 1987). Omega-3 fatty acids (including EPA) have been found to play an important role in prevention of degenerative and cardiovascular diseases (Hooper et al. 2004; Ross et al. 2007). Moreover EPA has been reported to be part of the cell membrane of microalgae and to play an important role in cellular repair and membrane development in microalgae (Huerlimann et al. 2010; Valentine and Valentine 2004). Thus this can also be one of the reasons that *Nannochloropsis* sp. *cells* were still intact at higher UV-C dosage of 1000 mJ/cm^2 as cells had relatively high concentration of EPA (Fig. 5) when compared to the previous study (Sharma et al., 2014). Whereas, in previous study on *Tetraselmis* sp, it was reported that UV-C induces antioxidants activity in microalgal cells and as EPA has strong affinity to absorption to ROS (Bouhamidi et al. 1998), an increase in EPA was assumed as a defence mechanism of microalgal cells.

In conclusion, this study highlights the use of low exposure of UV-C radiation to induce lipids, particularly EPA, within 24 h of the treatment. This presents a significant improvement to lipid induction by nutrient starvation only. The optimized large-scale

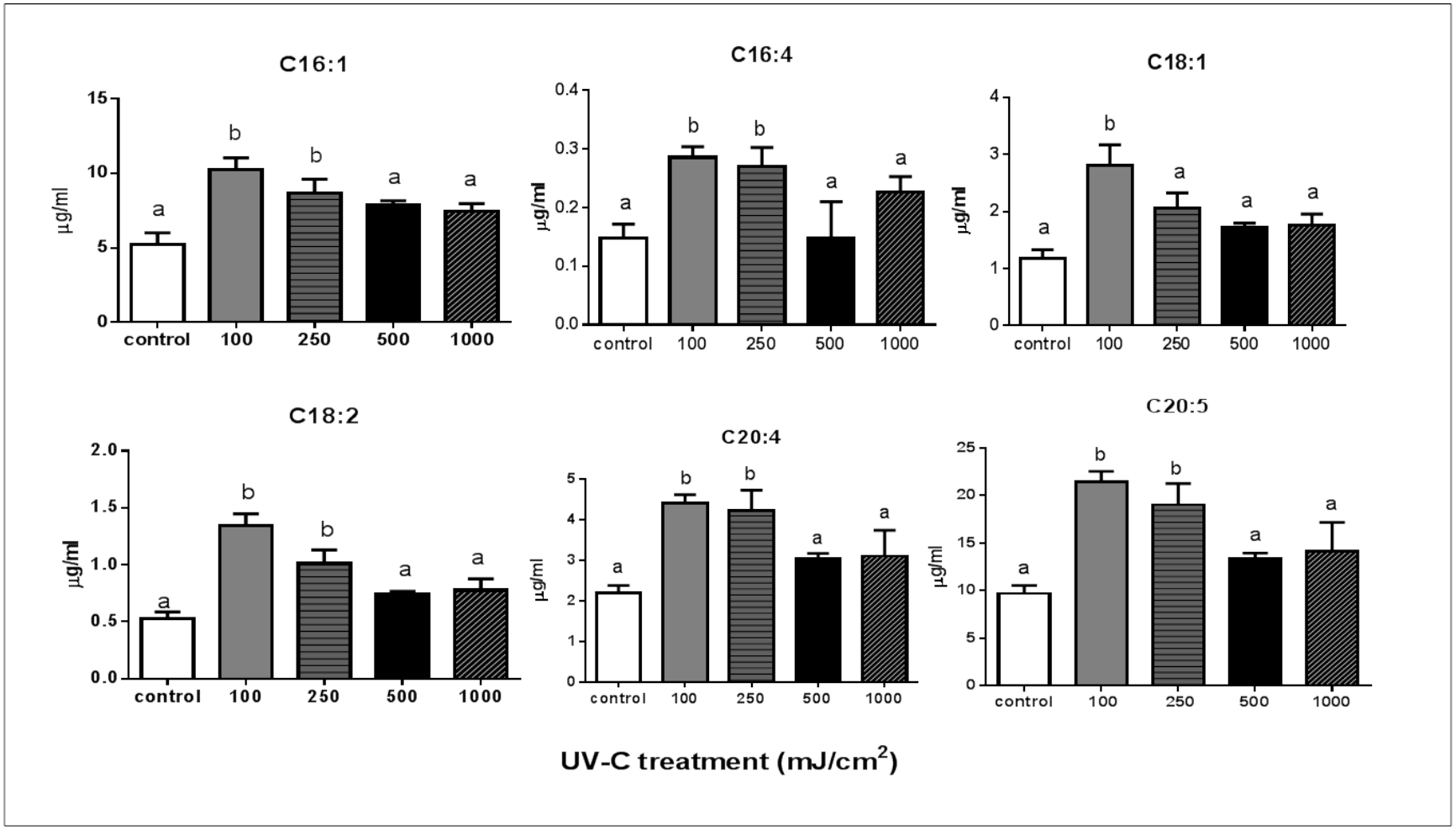
production of EPA in *Nannochloropsis* may provide a much-needed alternative, vegetarian, and land-based source of omega-3.

Acknowledgements

We wish to thank the Australian Research Council for financial support.



Supplementary Fig. 1: Comparison of different saturated fatty acids present in *Nannochloropsis* sp. BR2 following different treatments of UV-C radiation. Values are mean ±SE (n = 3); bars with different alphabets indicate significant differences (*P*<0.05).



Supplementary Fig. 2: Comparison of different unsaturated fatty acids present in *Nannochloropsis* sp. BR2 following different treatments of UV-C radiation. Values are mean ±SE (n = 3); bars with different alphabets indicate significant differences (P<0.05).

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Chapter 6: UV-C mediated rapid carotenoid induction and enhanced harvesting performance of Dunaliella salina and Haematococcus pluvialis (Submitted)

Overview

From the above studies conducted on C*hlorella* sp. BR2*, Tetraselmis* sp. M8 and *Nannochloropsis* sp. BR2, it was discovered that short exposure of UV-C radiation could significantly induce lipid biosynthesis, most importantly polyunsaturated fatty acids (PUFAs) and cause oxidative damage. As carotenoids have been proven to have high anti-oxidative activity and scavenge free radicals the hypothesis was to use a small dosage of UV-C radiation to induce carotenoids in *Dunaliella salina* and *Haematococcus pluvialis*., two microalgae that were found to contain high levels of beta-carotene and astaxanthin, respectively.

Key Findings

- A significant increase in total carotenoids and beta-carotene was observed after exposure to 50 mJ/cm^2 for *D. salina* and two-fold increase in total astaxanthin production was observed at 30 mJ/cm^2 for *Haematococcus* sp.
- UV-C radiation of 50 mJ/cm^2 was found sufficient to detach flagellate in both species tested and approximately 95% of settling was observed after 15 h and 3 h for *D. salina* and *H* pluvialis., respectively.
- This was the first study to report UV-C radiation not only induces lipids biosynthesis, but also induces carotenoids biosynthesis in *D. salina* and *H* pluvialis.

Chapter 6: UV-C mediated rapid carotenoid induction and enhanced harvesting performance of *Dunaliella salina* and Haematococcus *pluvialis* (Submitted)

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PMS and YL have contributed equally.

Abstract

Microalgae are primary producers of organic matter in aquatic environments and are a promising source of a wide diversity of compounds, such as carotenoids and essential fatty acids. However, commercial-scale microalgae application is rarely economical due to slow-growing high carotenoid-producing microalgal strains and high harvesting costs. Here, we present a novel UV-C radiation technique to not only induce lipid accumulation but also carotenoid biosynthesis and natural settling in *Dunaliella salina* and *Haematococcus pluvialis*. Maximum total carotenoid and β-carotenoid inductions were achieved following 50 mJ/cm^2 of UV-C radiation for *D. salina*, whereas a five-fold increase in total astaxanthin production was achieved at 30 mJ/cm^2 in *H. pluvialis*. Meanwhile, a radiation of 50 mJ/cm^2 was sufficient to detach algal flagella in both tested species. Approximately 95% of algal cells settled after 15 h and 2 h for *D. salina* and *H. pluvialis,* respectively.

Introduction

Microalgae are considered as a potential feedstock, not only for second generation biofuels, but also for the production of a wide diversity of compounds, ranging from value-added products, such as carotenoids, pharmaceuticals and nutraceuticals (Li et al. 2007; Olaizola 2003; Pérez-López et al. 2014; Schenk et al. 2008; Sheehan et al. 1998). Microalgae are the primary producers of organic matter in aquatic environments due to their robust photosynthetic activities. They also possess several advantages over

terrestrial plants: they are relatively easy to cultivate, do not need to compete with food production, can adapt to some extreme environmental conditions by producing a variety of secondary metabolites, and can also be used as efficient bioremediation tools, for instance for sequestering CO_2 from streams derived from industrial processes (Ahmed et al. 2014; Munir et al. 2012; Olaizola 2003; Suh et al. 2006). Therefore, microalgae have become a promising natural feedstock in the new century. As the cost-effectiveness of algal biodiesel has been challenged, there is mounting interest on high-value bio-compounds produced from microalgae. The typical representatives are *Dunaliella salina* and *Haematococcus pluvialis* that can produce a high level of β-carotene and astaxanthin, respectively.

Carotenoids are generally non-polar, lipid-soluble organic pigments that give microalgae yellow, orange and red colour due to carotenoids absorbing visible light. Carotenoid pigments have high anti-oxidation properties, so they functionally play an active role in protecting the photosynthetic apparatus by dissipating excess energy (Ahmed et al. 2014; Stahl and Sies 2012). They also play a major role in photosynthesis by harvesting light and stabilising protein folding in the photosynthetic apparatus (Choudhury and Behera 2001). Due to their long chain carbon and hydrogen bonds, carotenoids can scavenge singlet oxygen which mainly arises from sunlight absorption by chromophores and thus protect chlorophylls, lipids, proteins and DNA from oxidative damage (Ahmed et al. 2014; Ibañez and Cifuentes 2013). Furthermore, carotenoids have been used as feed for aquaculture and as colorants by the food industry. In recent years, they have also become popular as dietary supplements due to the findings that carotenoids are powerful antioxidants and therefore can provide protection against ailments, such as cardiovascular diseases, certain cancers, age-related macular degeneration, and neurodegenerative diseases e.g. amyotrophic lateral sclerosis (Ibañez and Cifuentes 2013). *Haematococcus* algal meal serves as a colour additive in salmonid feeds, and also is a health-proven dietary-supplement for human consumption (Guedes et al. 2011). Therefore, microalgae *D. salina* and *H. pluvialis* have attracted a number of researchers' interests as valuable carotenoid producers and also entrepreneurs' attention in the last decade.

It is well known that microalgae can modify the lipid biosynthesis pathway and redirect it to produce some secondary metabolites upon environmental stress conditions (e.g. nutrient deprivation, light limitation, UV irradiation, pH, temperature; Aflalo et al. 2007; García-Malea et al. 2009; Shahid and Mohammad 2013; Sharma et al. 2012, 2014). Hence, many studies were conducted on optimisation of culturing condition, high temperature and salinity stress on *D. salina* to optimise β-carotenoids production (Fernández-Sevilla et al.

2010; Herrero et al. 2006; Kleinegris et al. 2011). Similarly, the production of astaxanthin in *H. pluvialis* has been extensively studied, typically with the aim to increase yields (Domınguez-Bocanegra et al. 2004; García-Malea et al. 2009; Olaizola 2000). The stress conditions used to induce carotenoid production in these microlagal strains include a combination of high salinity (for β-carotene), nutrient deprivation, high light, osmotic shock (for astaxanthin) at a stage when cultures are in late exponential growth phase (Ahmed et al., 2013). These attempts have resulted in the accumulation of up to 4% (w/w) astaxanthin and 14% (w/w) β-carotene in the dry biomass weight in *H. pluvialis* and *D. salina* (Borowitzka, 2013).

However, due to lengthy processes of these induction methods, the development of new efficient techniques is warranted that can significantly reduce the time for high-level carotenoid accumulation in *H. pluvialis* and *D. salina*, thereby improving cost-effectiveness of production. Synthesis and accumulation of large amounts of carotenoids in microalgae can be achieved by either acting individually or by applying a combination of stresses, including nutrient stress, radiation, pH and temperature change, heavy metals and other chemicals, and also by some metabolic engineering approaches (Gao et al. 2013; Renaud et al. 2002; Gao et al., 2014). However, it typically takes 3-5 days until significant amounts of carotenoids are synthesised which is accompanied by slow growth rates and thus finally affects the total algal biomass and carotenoid productivities. More importantly, modification of temperature, pH, salinity and/or heavy metals concentration are difficult to apply at large-scale. Additionally, the microalgae dewatering/harvesting process constitutes the most energy intensive and expensive component for carotenoid production from microalgae and becomes a major impediment for industrial scale manufacturing of *H. pluvialis* and *D. salina* (Garg et al., 2012; Ahmed et al., 2013; Garg et al. 2012). Therefore, it would be highly beneficial for the carotenoid production process if induction methods could simultaneously assist in the dewatering and harvesting process of carotenoid-induced *H. pluvialis* and *D. salina* cultures.

UV-A and UV-B have already been reported to increase fatty acid and carotenoid production in several microalgal strains but which typically takes more than 7 days (Srinivas and Ochs 2012; Goes, 1995). UV-C is particularly promising due to the fact that it carries more energy per photon. We have previously shown for *Tetraselmis* sp., that UV-C radiation acts a highly effective method, not only for inducing high-level fatty acid production but also leads to rapid settling of these flagellate microalgae (Sharma et al. 2014). This process was accompanied by an increase in reactive oxygen species (ROS)

and up-regulation of redox homeostasis-regulating enzymes, superoxide dismutase and glutathione S reductase (Sharma et al. 2014). As carotenoids have a high antioxidative activity and can scavenge free ROS, the rationale behind the present study was to test whether a low dosage of UV-C radiation can not only induce fatty acid synthesis, but also lead to rapid carotenoid production within 24-48 hours and whether prolonged exposure causes settling of flagellate *D. salina* and *H. pluvialis* cultures.

Materials and methods

Microalgal cultures and UV-C treatments

Microalgae *Dunaliella salina* (Lim et al., 2012) and *Haematococcus pluvialis* (Lim et al., 2012) were cultured in the Algae Biotechnology Laboratory at The University of Queensland. The isolate of *D. salina* was grown in f/2 medium in autoclaved 60 ppt saltwater, whereas *H. pluvialis* was cultured in BMM medium in autoclaved tap water (Bischoff et al., 1963). Primary stock cultures were maintained aerobically in 2.5 L Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, on an orbital shaker (Thermoline) at 100 rpm under a 12:12 h light/dark photoperiod of fluorescent white light (120 µmol photons $m^{-2}s^{-1}$). The cell density reached $1.7x10^6$/mL and $5x10^5$/mL for *D. salina* and *H. pluvialis,* respectively, prior to the UV-C radiation trials.

Nitrate and phosphate levels were determined using the corresponding API Nutrient testing kits, according to the manufacturer's instructions. After gently stirring, 25 mL aliquots of *D. salina* and *H. pluvialis* cultures were pipetted into Cole-Parmer sterile tissue culture dishes (150 mm), forming a thin layer inside a total of 18 plates, each for both cultures. Plates were randomly divided into six groups with three plates as replicates for UV-C radiation (253 nm) treatment in a UV chamber, with dimension of 31.7 x 24.1 x 15.2 cm containing five G8T5 format, minibipin bulbs (Biorad, Gs-Genelinker, California, USA).. Based on our preliminary tests, the radiation levels were set at 0, 50, 75, 100, 150 and 200 mJ/cm^2 for *D. salina* and subsequently incubated for 24 h, whereas *H. pluvialis* plates were exposed to 0, 30, 50, 100, 200 and 400 mJ/cm^2 and incubated for 48 h. Algae survival rates were measured by counting the live cells based on visibly intact chloroplasts in every 100 cells on each plate. The cell size was also measured by compound microscopy (Olympus).

Microscopic analyses

After the UV-C carotenoid induction phase, microalgae cells were stained with 2 µg/mL Nile red (dissolved in acetone; Sigma, USA) for 15 min and photographed using a fluorescent Olympus BX61 microscope fitted with a 100 W High Pressure Mercury Burner and an Olympus DP10 digital camera. Differential interference contrast and epifluorescent (excitation: 510–550 nm, emission: 590 nm) images were obtained at 20× magnification.

Lipid fluorescence analysis

For each replicate, 1 mL of algae cells was sampled and stained with 3 µL Nile red solution (a 10 mg/mL Nile red stock solution was prepared in acetone and stored in the dark at 4°C). After 20 min incubation in the dark, the lipid fluorescence intensity of cells was detected by plate reader fluorescent spectroscopy. A total of 200 µL of each sample was loaded into 96 well-plates and analysed with a Fluostar optima and Polarstar optima (BMG LAB tech) plate reader at excitation and emission wavelengths of 485/584 nm; the gain was set at 2400.

Settling of microalgae

After gently stirring, 25 mL aliquots of *D. salina* and *H. pluvialis* cultures were pipetted into a square Petri dish, forming a thin layer inside in a total of 18 plates for *D. salina and H. pluvialis,* respectively. Plates were randomly divided into six groups with three plates as replicates, and then exposed to UV-C radiation (253 nm) in a UV chamber as mentioned above. The cultures from the plate were then poured into 50 mL tubes and left undisturbed for settling. Cell count was taken at 2 and 15 h.

Analyses of fatty acid and carotenoid profiles

As described previously (Sharma et al, 2014), 4 mL of algal culture on each plate were centrifuged at 16,000xg for 3 min. The supernatant was discarded and the lipid in the algal pellet was hydrolysed and methyl-esterified with a 300 µL of 2% H_2SO_4 methanol solution at 80°C by shaking (480 rpm) for 2 h on a thermalmixer (Eppendorf). Prior to esterification, 50 µg of heneicosanoic acid (C21) were added to the pellet of each sample as an internal standard. After esterification, 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane (analytical grade) were added and vortexed for 20 s. Phase separation was achieved by centrifugation at 16,000xg for 3 min and the hexane layer was used for carotenoid profile

analysis. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out on an Agilent 6890 GC coupled to a 5975 MSD. A DB-Wax column (Agilent, 122-7032) was used with running conditions as described in Agilent's RTL DBWax method (Application note: 5988-5871EN). Identification of fatty acid methyl esters (FAME) was based on mass spectral profiles and retention times in the Agilent's RTL DBWax method. Each FAME was quantified using the formula:

Fatty acid (µg/mL) = (Total ionic current of fatty acid/ Total ionic current of standard) x (molecular mass of fatty acid/molecular mass of internal standard) x 50/4

For carotenoid extraction, the centrifuged algal samples were freeze-dried for 24 h. The carotenoid extraction was based on a method of Fanning et al. (2010). The concentrated carotenoid extract was dissolved in 2.5 mL of methanol/dichloromethane (50/50, v/v) for HPLC analysis. The gradient of mobile phases in high performance liquid chromatography (HPLC) analysis was set as: 0 min, 80% phase A and 20% phase B; 48 min, 20% A and 80% B; 49 min, 80% A and 20% B; 54 min, 20% A and 80% B (phase A - 92% methanol/8% 10 mM ammonium acetate; phase B - 100% methyl tert-butyl ether). A mass spetrometry scan was undertaken between 530 – 610 mass units in the APCI+ mode (Fu et al. 2012) using an Acquity UPLC H-Class system connected to a Quattro Premier triple quad (Micromass MS Technologies, Waters Corporation, Milford, MA, USA). Source temperature and probe temperature were 150˚C and 600˚C, respectively, while desolvation and cone gas flow were at 450 and 50 L h^{-1}. The corona, cone and extractor voltages were 5.0 µA, 30 V and 3 V, respectively. Carotenoids were identified by their specific retention times, UV/Vis spectra and mass spectra against authentic standards (Lu et al. 2009). The concentrations of the identified carotenoids were determined using individual calibration curves.

Malondialdehyde assay in *Haematococcus pluvialis*

To better understand the underlying mechanisms for the observed shift of fatty acids towards unsaturated fatty acids following UV-C treatment, Thiobarbituric Acid Reactive Substances (TBARS) assays were carried out on *H. pluvialis*. Decomposition of unstable peroxides derived from PUFAs resulting in the formation of malondialdehyde, were quantified colorimetrically following their controlled reaction with TBARS.

Data analysis

Data for lipid and carotenoid productivity was statistically analysed by one-way analysis of variance (ANOVA) with separately-grown microalgal cultures as the source of variance and growth rate or lipid or carotenoid productivity as dependent variables. This was followed by Turkey's multiple comparisons test where appropriate (P<0.05). Student's t-test was used for pair-wise comparisons.

Results

UV-C exposure induces settling of *D. salina* and *H. pluvialis* cultures

To test whether nutrient starvation and UV-C treatment can lead to enhanced increases in carotenoids biosynthesis, combined sequential stress treatments were carried out on *D. salina* and *H. pluvialis*. For *D. salina,* the cell survival rate sharply declined with an increase of UV-C radiation (Fig 1a). The survival rate of *D. salina* was about 50% (LD50) at 50 mJ/cm^2, whereas there was only 1-3% of survival rate at 200 mJ/cm^2. Radiation of 50 mJ/cm^2 was sufficient to detach the flagella and induce settling for almost 100% of the cells within 15 h, but reached 50% in the first 2 h post treatments (Fig 1b).

Similarly, *H. pluvialis* cultures were radiated at different dosages of UV-C radiation. There was a linear decline of cell survival rates in *H. pluvialis,* reaching 50% (LD50)at 100 mJ/cm^2, and dropped to 30% at 200 mJ/cm^2 (Fig 1a). However, the cell survival rate did not further decrease between 200 and 1000 mJ/cm^2 (Fig 2a). Similar to *D. salina,* 50 mJ/cm^2 could effectively detach the flagella of *H. pluvialis*, and approximately 75% settling was achieved at this dose, but almost 99% of the *Haematococcus* cells settled at 150 mJ/cm^2.

UV-C irradiation induces microalgal lipid biosynthesis in *D. salina* and *H. pluvialis*

Nitrogen-deprived cells of *D. salina* and *H. pluvialis* cultures showed a clear increased amount of lipid droplets within 24 h of UV-C radiation (Fig. 2 and Fig. 3a-e). This is consistent with a recent study on a different UV-C-treated green alga, *Tetraselmis* sp. (Sharma et al. 2014). With increase of UV-C radiation dosage from 50 to 200 mJ/cm^2, the lipid fluorescence intensity inside the cells became stronger for both microalgae. Upon dosages higher than 200 mJ/cm^2, algal cell bleaching and rupturing became apparent in

D. salina. cells also displayed more clumping (arrows on Fig 2d) upon UV-C exposure of 50 mJ/cm^2 or higher in *D. salina* (Fig 2c-d).

In *H. pluvialis,* both, 30 and 50 mJ/cm^2, were sufficient to induce lipid droplets and the value became stabilised when UV-C radiation was higher than 50 mJ/cm^2. In *D. salina,* 50 and 75 mJ/cm^2 of radiation were more effective for lipid induction, demonstrated by a significant increase of lipid florescence inside the cells (P<0.05, Fig 2e). However, both microalgae showed no significant difference on cell mortality between the control and treated cultures when exposed to higher than 100 mJ/cm^2 of UV-C radiation.

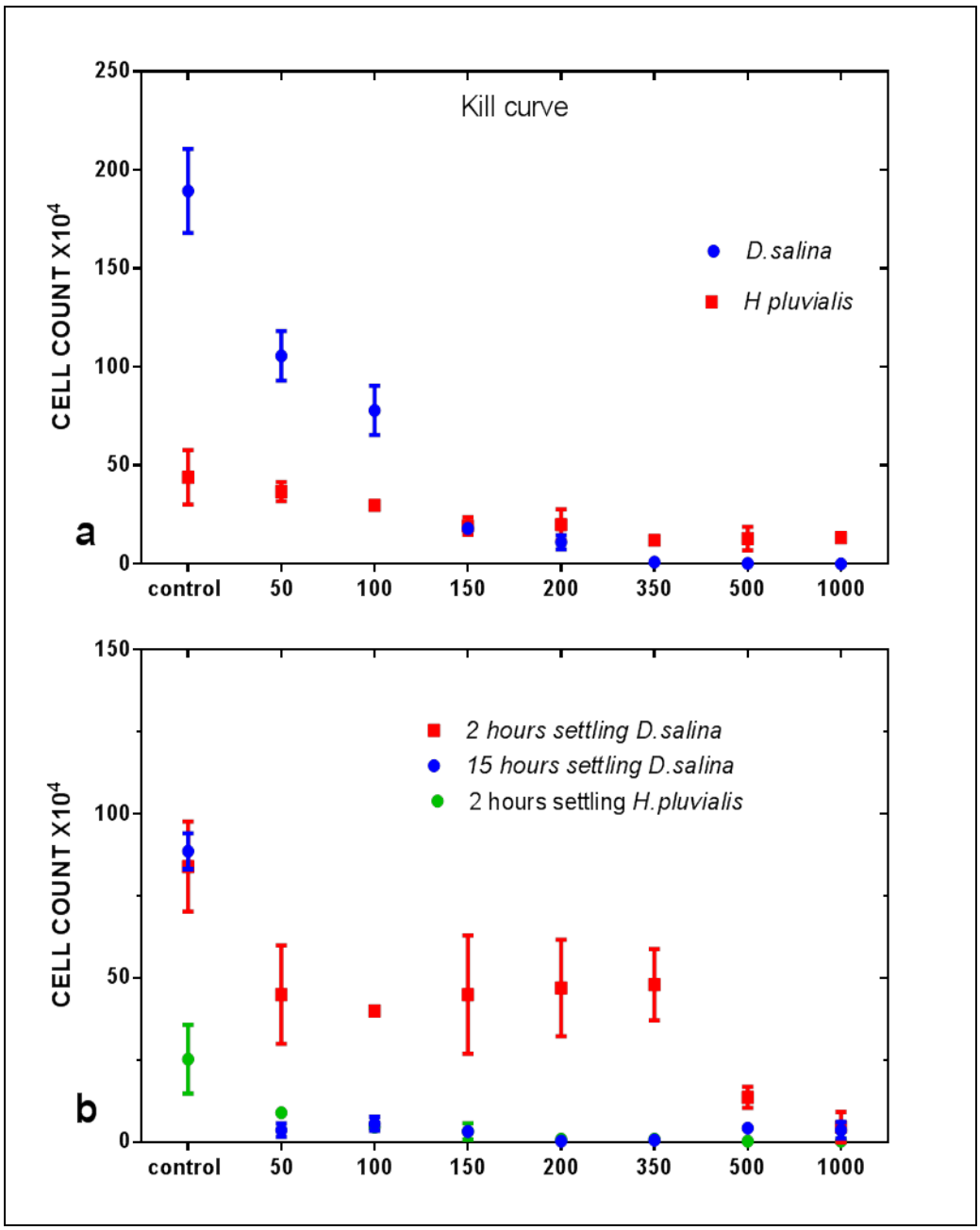

Figure1. a) Kill curve of *D. salina and H. pluvialis* cells, showing the number of survival cells after different UV-C radiation dosages; b) Settling curve of *D. salina* and *H. pluvialis*. Shown are mean values ± SEs from three independent treatments of separately-grown algal cultures, each.

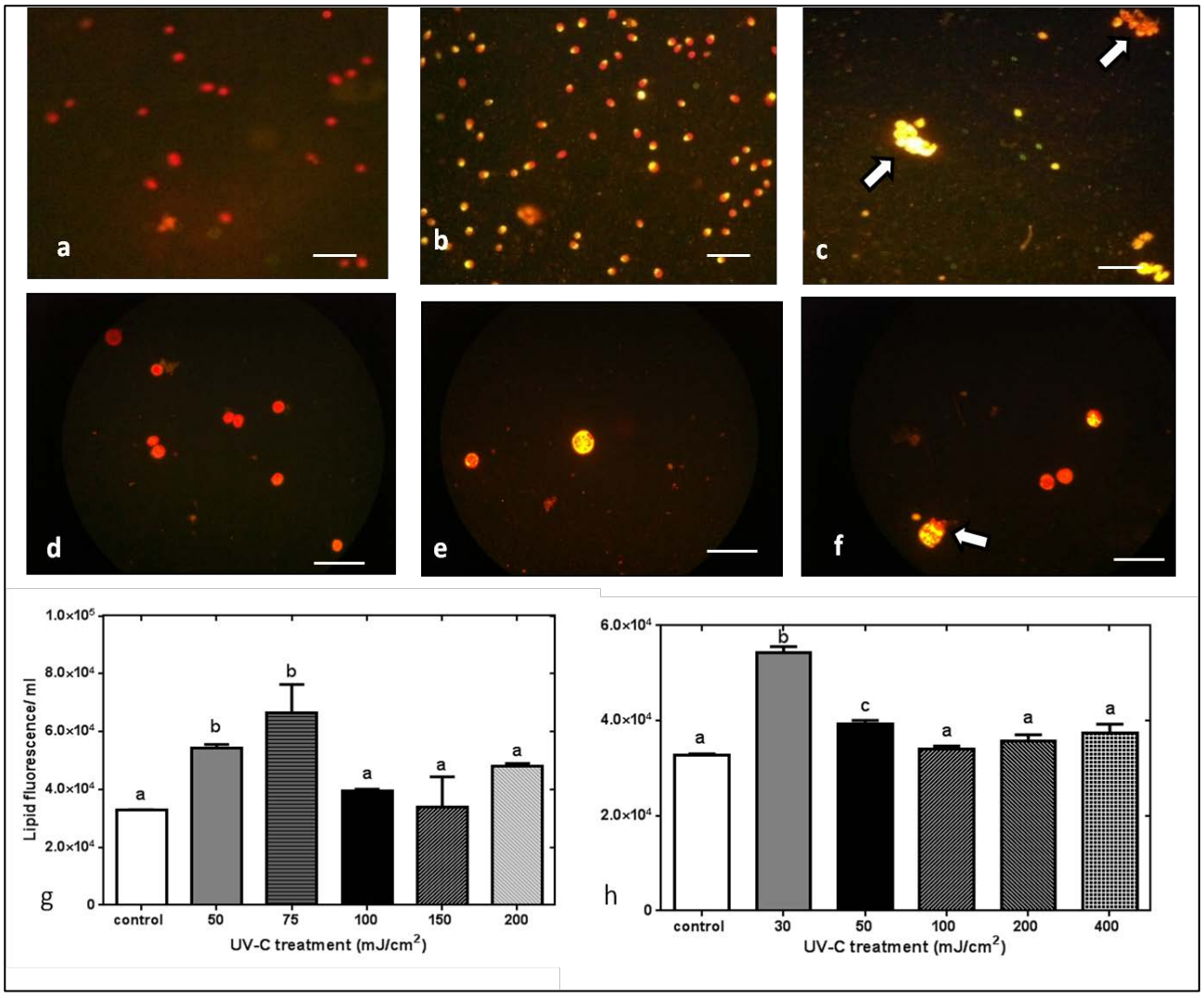

Figure 2. a-c) Nile red-stained cells of *D. salina* cultures exposed to UV-C radiation at 0 (control), 75 and 100 mJ/cm^2, respectively. d-f) Nile red-stained cells of *H. pluvialis* cultures exposed to UV-C radiation at 0 (control), 30 and 100 mJ/cm^2, respectively. g,h) Lipid fluorescence analysis of different UV-C treated samples of *D. salina* and *H. pluvialis*.

UV-C treatment leads to increased β-carotene and astaxanthin biosynthesis

Carotenoid contents were measured in microalgal cells to test whether UV-C exposure can stimulate biosynthesis of these anti-oxidative compounds. At 24 h after exposure to 50 mJ/cm^2, there was a clear increase of total carotenoids content in *D. salina* (P<0.05, Fig. 3a). As a major component, β-carotene content nearly doubled compared to the mock-treated control cultures (P<0.05). However, when UV-C radiation was higher than 50 mJ/cm^2, both total carotenoids and β-carotene content declined sharply and dropped back to the control levels. Apart from β-carotene, the mean value of lutein content was also higher than the control at 50 mJ/cm^2 but was not statistically significant (P>0.05, Fig. 3c).

In *H. pluvialis,* a significant increase of astaxanthin production was obtained at 30 mJ/cm^2 (*P*<0.05,Fig. 4a-g), but gradually declined with an increase of UV-C radiation (≥ 50 mJ/cm^2) and became similar to the control level (Fig. 4d). Three isomers of astaxanthin were detected in this study, showing about three times less of cis-astaxanthin content than either di-astaxanthin or mono-astaxanthin in tested *H. pluvailis* cells (Fig. 4d-g). With increased UV-C radiation, these astaxanthins displayed a similar fluctuation pattern as total astaxanthins, but only mono-astaxanthin was still significantly higher than in the control cultures at 50 mJ/cm^2 (*P*<0.05). Microscopy analyses revealed that some cells had burst open and cell debris was observed (Fig. 2f).

UV-C treatment induces polyunsaturated fatty acid production and malondialdehyde activity

Although lipid florescence analysis showed a large of amount of lipid droplets induced in UV-C treated *D. salina* cells, GC-MS data did not show any significant increase in total fatty acid determination. Only a slight increase was detected in the cells radiated at 75 mJ/cm^2 of UV-C treatment (Fig. 5a). However, the total unsaturated fatty acid content significantly increased almost two-fold at 75 mJ/cm^2 (*P*<0.05, Fig. 5b). By comparison, the content of total saturated fatty acids was unaltered in the tests (Fig. 5c).

It appears plausible that UV-C stress leads to cellular lipid biosynthesis to improve survival of microalgal cells. Figure 4a shows that as the UV-C radiation increased from 0 to 1000 mJ/cm^2 the formation of malondialdehyde increased in *H. pluvialis* cells and reached the highest level at 1000 mJ/cm^2 (fig 6), indicating that UV-C-treated cells underwent oxidative stress and likely caused damage to DNA and cellular membranes. Hence the increased amount and proportion of astaxanthin in UV-C-stressed cells may be required for providing sufficient antioxidant capacity and repairing cellular damage to restore redox homeostasis in the cells.

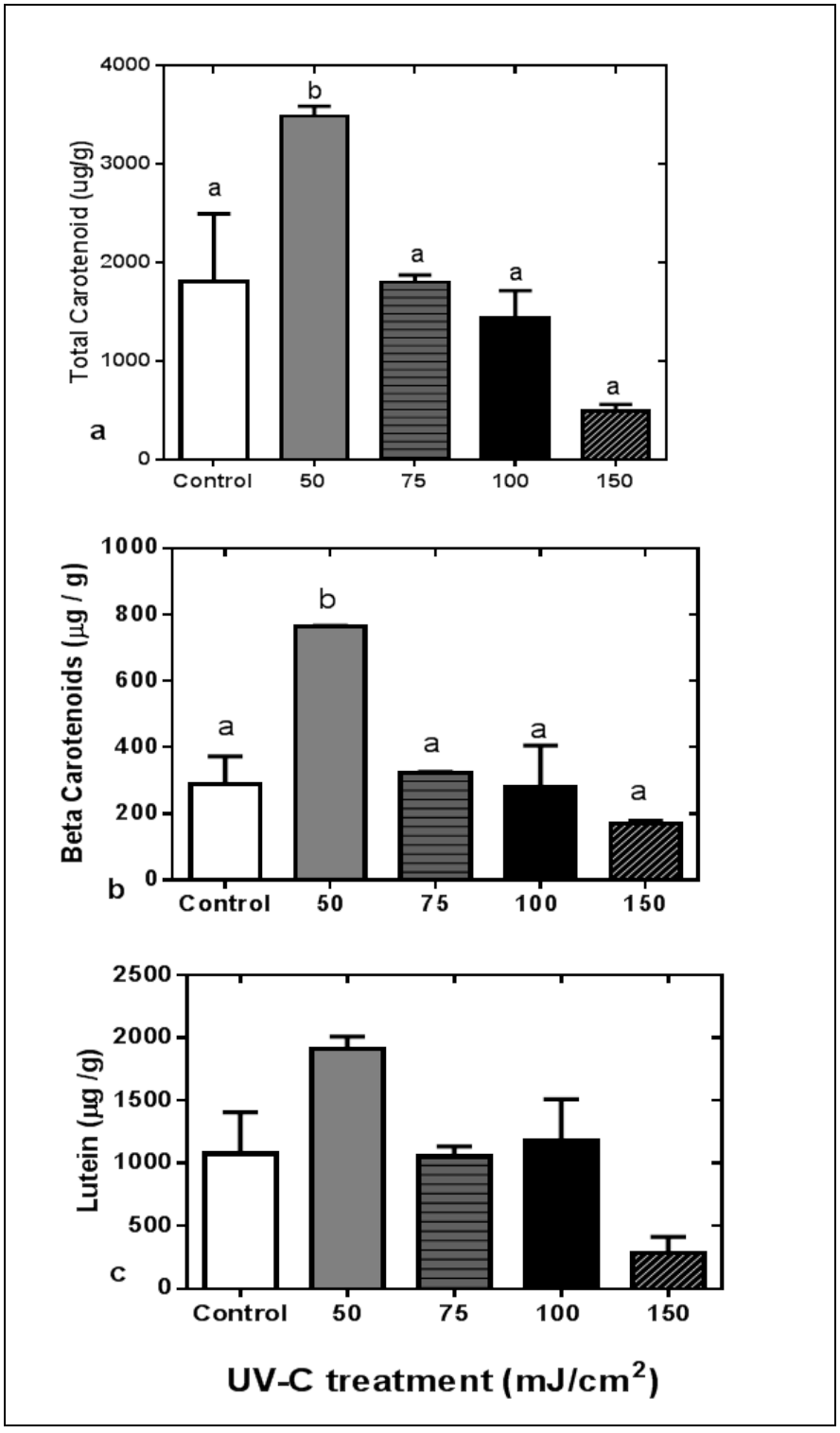

Figure 3. a-c) Total carotenoids, β-carotenoids and lutein measured in *D. salina* cells following different UV-C treatments. Shown are mean values ± SEs from three independent treatments of separately-grown algae cultures.

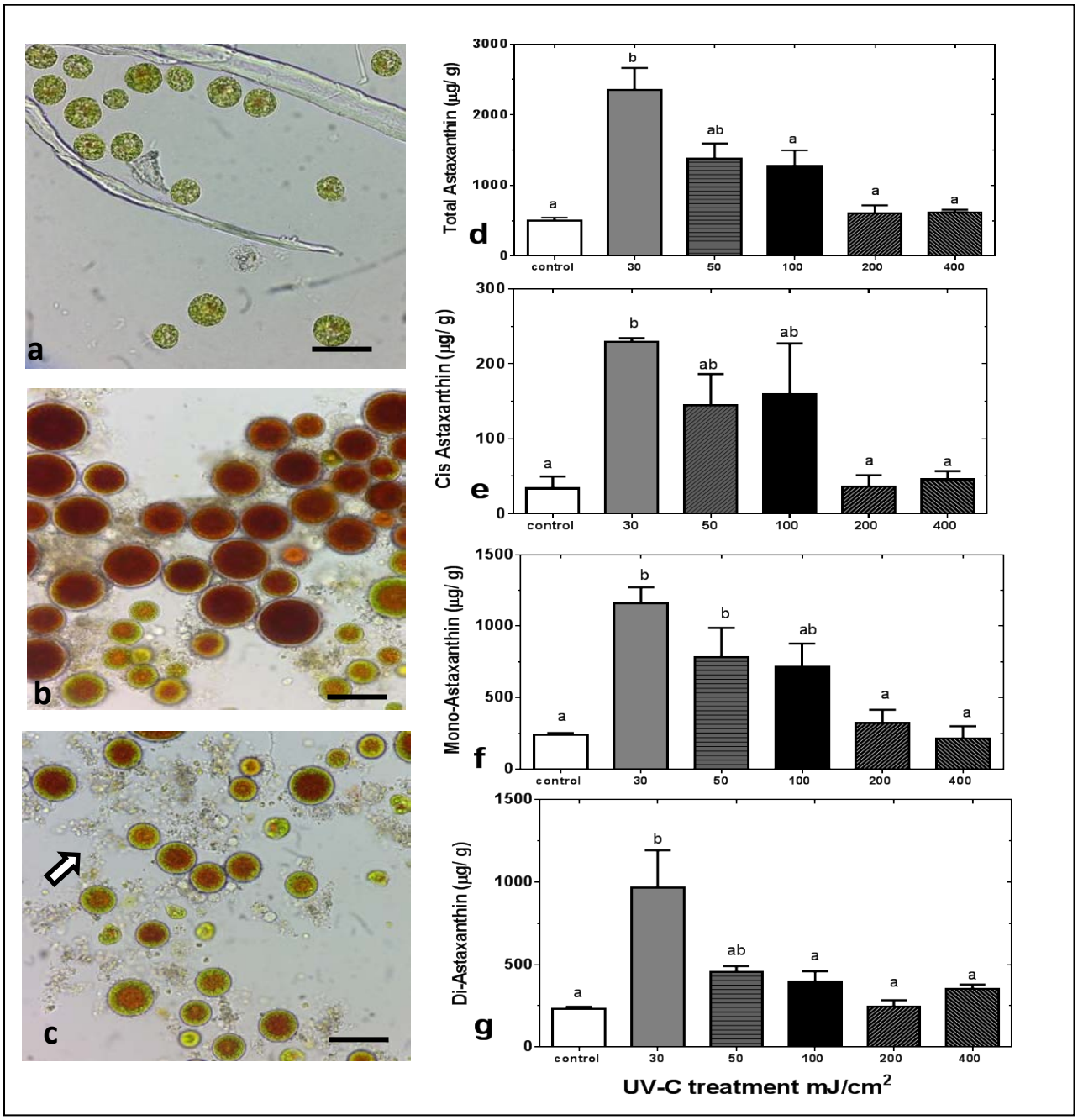

Figure4: a-c) Visible carotenoid induction in UV-C-treated *H. pluvialis* cultures at 0 (control), 30 and 100 mJ/cm^2, respectively. d-g) Total astaxanthin, cis-astaxanthin, mono-astaxanthin and di-astaxanthin contents in *H. pluvialis* cells upon different UV-C radiations. Shown are mean values ± SEs from three independent treatments of separately-grown algae cultures. Different small letters represent significant differences between treatments (P<0.05).

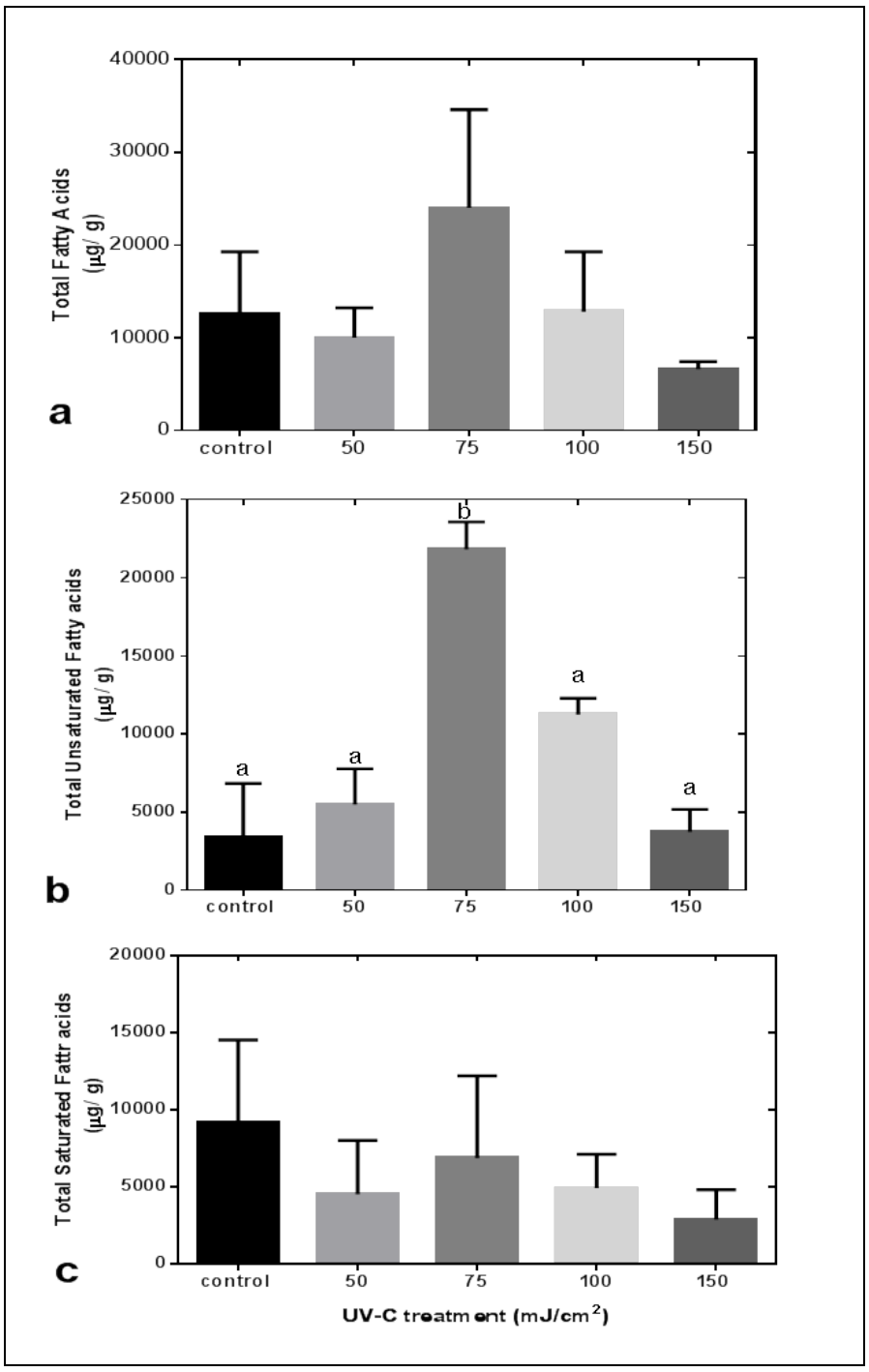

Figure 5 :a-c) Total fatty acids, total unsaturated fatty acids and total saturated fatty acids induced in *D. salina* cultures at different dosages of UV-C radiation. Shown are mean values ± SEs from three independent treatments of separately-grown *D. salina* cultures. Different small letters represent statistically significant differences between treatments (P<0.05).

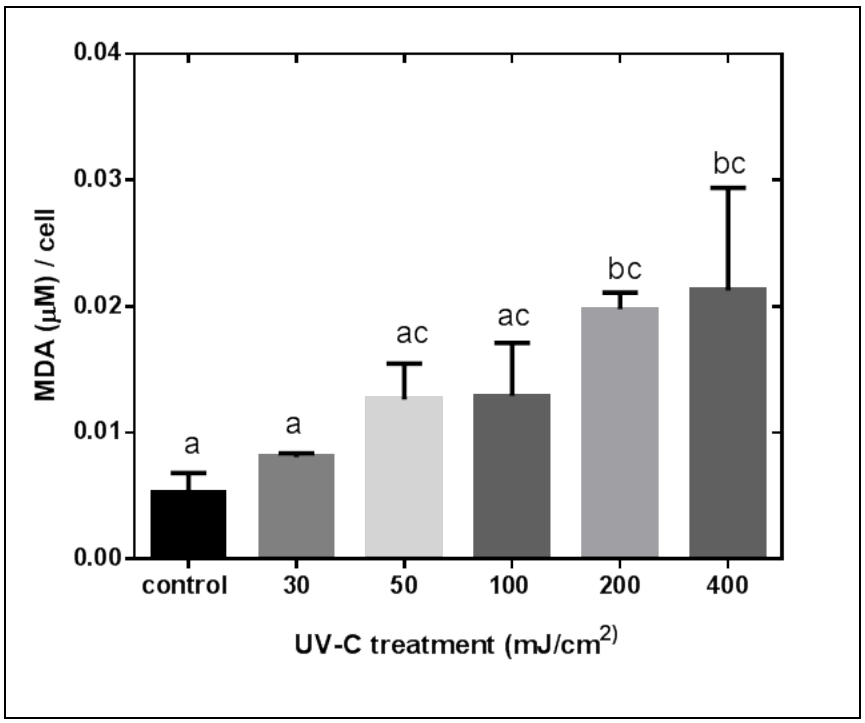

Figure 6: Decomposition of unstable peroxides (TBRAS) assay of UV-C-stressed and mock-treated *H. pluvialis* cells at different dosages. Values are mean ± SEs from three separately-grown and -treated cultures. Different small letters represent statistically significant differences between treatments (P<0.05).

Discussion

To our knowledge, this is the first study to report that UV-C radiation can induce both lipid and carotenoid biosynthesis in *D. salina* and *H. pluvialis*. In particular, this study highlights the effectiveness of using low dosage of UV-C radiation to stimulate the production of valuable carotenoids which only took 24 h for *D. salina* and 48 h for *H. pluvialis*, and also provides a cost-effective way to pre-concentrate microalgae. Maximum amounts of total carotenoids and β-carotene were obtained at 50 mJ/cm^2 in *D. salina.* About a five-fold increase of total astaxanthin production was measured following UV-C treatment at 30 mJ/cm^2 in *Haematococcus* cells. However, 50 mJ/cm^2 of UV-C radiation was found sufficient to detach flagella in both microalgal species and approximately 95% of settling rate was achieved after 15 h and 2 h for *D. salina* and *H. pluvialis*, respectively.

Probably caused by the lack of rigid cell walls, *D. salina* showed a high level of cell mortality at 100 mJ/cm^2 of UV-C radiation and almost 100% of the cells were dead up to 1000 mJ/cm^2 (Fig 1a). In contrast, *H. pluvialis* is known to have a rigid cell wall and turns into cysts during stress conditions. This is reflected in the reduced cell mortality for *H. pluvialis* in this study. Interestingly, a significant increase in lipid florescence was observed

in *H. pluvialis* at 30 and 50 mJ/cm^2, whereas it was at 75 mJ/cm^2 on *D. salina.* It is likely that this was associated with the cell density because *D. salina* (1.8×10^6/mL) was used at a much higher cell concentration than *H. pluvialis* (5 ×10^5/mL). However, the report on *Tetraselmis sp*. showed that UV-C radiation of 100 mJ/cm^2 was optimal to induce significant amounts of lipids, based on a similar cell density (1.7×10^6/mL; Sharma et al. 2014) as our tested *D. salina* cultures. Clearly, the effectiveness of UV-C-mediated lipid induction is dependent on the structure and dimension of target algal cells.

In the present study, a significant increase of total carotenoids and two-fold increase of β-carotene in *D. salina* were found after 50 mJ/cm^2 UV-C treatments within 24 hours. This is consistent with a study on *Dunaliella bardawil* with UV-A radiation (320–400 nm), resulting in a two-fold induction of β-carotene in 24 days (Mogedas et al. 2009). As lutein production did not fluctuate significantly following UV-C exposure, it is conceived that β-carotene could be more active than α-carotene to deal with radiation stress in *Dunaliella* microalgae. This role seems to be taken by astaxanthin in UV-C-stressed *H. pluvialis* cells as discussed below.

Commercial production of astaxanthin by *Haematococcus* sp. has been implemented by microalgae companies, like Cyanotech, Algaetech and Aquasearch. These companies use a two-stage cultivation system, consisting of a first step to produce green biomass under optimal growth conditions (green stage), followed by a second stage when the microalgae are exposed to adverse environmental conditions to induce accumulation of astaxanthin (red stage) (Guerin et al. 2003). In the present study, once the culture density had reached an exponential phase (green stage), cultures were irradiated with different dosages of UV-C and a five-fold induction of total astaxanthins was detected following treatment at 30 mJ/cm^2 within 48 h, which reached up to 2.7 mg/g (2.7 mg/l) of astaxanthin compared to 0.5 mg/g of control level (Fig. 4d). As the tested *Haematococcus* cells were not fully transformed into cysts in this study, the increased astaxanthin level was below the astaxanthin contents in large-scale facilities which are typically 2.2 mg L^{-1} —even though maximum astaxanthin productivities of 11.5 mg L^{-1} d^{-1} can be attained at bench scale (Olaizola, 2000). Future studies may focus on maximising astaxanthin productivity by combining UV-C stress with other cyst-inducing treatments.

In the present study, UV-C radiation induced more unsaturated fatty acids, than saturated fatty acids. This is similar to the results obtained for *Tetraselmis* sp. (Sharma et al. 2014). In microalgae, the deleterious effect of UV light on thylakoid membrane integrity

leads to the production of ROS and PUFAs have a strong affinity or absorption to ROS (Gupta et al. 2008). Therefore, the increment of PUFAs can be interpreted as a defence response against UV-C-generated cellular ROS. This inference is also coincident with MDA determination which gradually increased with UV-C radiation. It has been reported that ROS play a crucial role in triggering synthesis of carotenoids, as part of a cellular strategy aiming for cell protection against oxidative damage (Ip and Chen 2005; Salguero et al. 2003). Thus in *H. pluvialis,* the increased MDA concentration indicates that UV-C-treated cells were synthesising astaxanthin to prevent oxidative damage. Other studies have reported that flashing light increased the rate of astaxanthin production in *H. pluvialis* by at least four-fold relative to that under continuous light sources (Fábregas et al. 2001; Kim et al. 2006). However, the effect of light irradiance depends on several factors, such as culture density, cell maturity (flagellates are much more sensitive than palmelloids) (Wang et al. 2003). A low UV-C radiation of 30 mJ/cm^2 was sufficient for *H. pluvailis* to transform quickly into the palmelloid/apalanospore stage, thus providing a short cut for astaxanthin accumulation. The information derived from this study suggests that further studies using UV-C for astaxanthin induction would be best focused on the aplanospore stage of *Haematococcus*, to investigate the effectiveness and evaluate commercial contribution of rapid UV-C induction to commercial *H. pluvialis* cultivation.

One of the highlight of this study was that, UV-C not only induced total carotenoid contents but that it also bridged the gap between the time taken to harvest the induced cells. The optimum carotenoids induction dosage of 50 mJ/cm^2 was found to be sufficient to detach the flagella of both the species tested. Moreover, given that the fragile cell structures of *D. salina* poses a problem for using high- speed centrifuges, UV-C mediated settling might present a good alternative for primary dewatering. Finally it should be mentioned that all the results obtained in this study were obtained from lab-scale cultivation. Further optimisation of UV-C treatment might lead to the development of rapid and cost-effective protocols of induction and harvesting of microalgae in commercial cultivation systems.

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Conclusion and Future direction

Microalgae cultivation in a biorefinery concept requires optimal and cost-effective production of multiple product streams, including high-value carotenoids and omega-3 fatty acids (e.g. for nutraceuticals), as well as triacylglycerides as biodiesel feedstock. Methods for the induction of lipid and carotenoid biosynthesis as well as cost effective harvesting technology are vital for successful microalgae cultivation in a biorefinery module. After considering different lipid induction (Chapter 1) and harvesting (Chapter 2) techniques it was concluded that, lipid induction with light irradiation is a potential method to serve this purpose. Hence a novel technique, LIS (Lipid induction and settling) was developed that can not only induce lipids in exponential phase within hours, but also bridges the gap between the time taken in microalgae biomass production to harvesting the biomass.

In the first study of UV-C radiation conducted on *Chlorella* sp. BR2, it was found that along with rapid (within 24 h) lipid induction, the algal cells were also relatively altered. While the cell size gradually increased, the cell survival rate significantly declined by UV-C radiation ranging from 0-1000 mJ/cm^2. This study highlighted the efficiency of UV-C radiation on microalgae lipid induction, and also provided a benchmark for rapid lipid analysis by FACS. One of the major finding in this study was a significant increase in total unsaturated fatty acid content in the cells when compared to total saturated fatty acids at low UV-C radiation.

To test whether both, nutrient starvation and UV-C treatment can lead to further lipid biosynthesis and facilitate settling in flagellate microalgae, combined sequential stress treatments were carried out on *Tetraselmis* sp. M8. The procedure essentially separates biomass growth from lipid accumulation and harvesting, and was optimised to be completed within 48 h for *Tetraselmis* sp. M8, using pilot-scale outdoor cultivation. Interestingly, the decrease of saturated fatty corresponded to the increase of unsaturated fatty acids like which coincided with the results obtained for *Chlorella* sp.

To investigation whether, UV-C radiation could be used to induce high value fatty acids in *Nannochloropsis* sp. BR2. in particular the production of eicosacenoid pentanoic acid (EPA), an important fatty acid with proven health benefits. Combined sequential stress treatments were carried out to achieve this aim. The UV-C treated culture showed significant increase for all detected USFA, most notably C20:5 (EPA) which accounted to be nearly 50 % of the total fatty acid.

In studies conducted on different microalgae it was discovered that short exposure of UV-C radiation could not only significantly induce lipid biosynthesis but also leads to increase in ROS production followed by up-regulation of enzymatic activity of superoxide dismutase (SOD), glutathione reductase (GR) and MDA, indicating high oxidative damage. As carotenoids have been proven to have high antioxidation activity and scavenge free ROS the later experiments were conducted on *D. salina* and *Haematococcus* sp to induce carotenoids. Significant increase in total carotenoids and beta-carotenoids induction was observed in *D. salina* and a twofold increase in total astaxanthin production was observed in *Haematococcus sp*. Whereas, radiation of 50 mJ/cm^2 was found sufficient to detach flagella in both the species tested and approximately 95% of settling was observed after 15 h and 3 h for *D. salina* and *Haematococcus* sp., respectively. This was the first study to report UV-C radiation not only induces TAG biosynthesis, but also carotenoids biosynthesis in *D. salina* and *Haematococcus* sp. It highlights the potential use of low dosage UV-C radiation to induce carotenoids within 48 h of treatment and also provides a cost-effective way to settle microalgae for easy dewatering and harvesting. The below summary shows the results for the different microalgae tested.

Table 1- Summary of different microalgae tested by UV-C lipid induction and settling (LIS) method. Red colour text indicates that the method has been optimised for pilot scale.

Microalgae species tested	Optimum UV-C Dose	Targeted induced compounds	Settling induced
(BR2) *Chlorella sp*	100 mJ	C18:3	n/a
Nanochloropsis sp	100-250 mJ	C20:5 (EPA)	n/a
(M8) *Tetraselmis sp*	100 mJ	C16:4	Yes
D. Salina	50 mJ	Total Caroteniods	Yes (3-15 hr)
Haematococcus sp	20 – 30 mJ	Astaxanthin	Yes (2 hr)
D. Salina (lab)	50-100 mJ	-	Yes (2-15 hr)
P. Lutheri	150- 250 mJ	-	Yes

Future directions

Large-scale optimisation of LIS treatment at demonstration facility, Pinjarra Hills UQ.

Future studies are well-positioned to take the findings of this thesis to commercial levels of large-scale production of microalgae, where the use of UV-C could assist in lipid biosynthesis, in particular high-value fatty acids, such as EPA, carotenoid biosynthesis and settling of flagellate microalgae. During the present study, a 250,000-L algae demonstration farm was constructed in Pinjarra Hills adjacent to the Brisbane River to develop large-scale production. Preliminary tests showed that UV-C exposure in pipes maybe the best option for cost-effective induction of lipids and/or carotenoids and settling for microalgal biomass harvesting.

Gene expression analysis and transformation.

In studies conducted on C*hlorella* sp. BR2*, Tetraselmis* sp. M8, *Nannochloropsis,* sp,. BR2, *D. salina* and *Haematococcocus* sp*.,* it was discovered that short exposure of UV-C radiation could significantly induce lipid biosynthesis, more importantly PUFAs and carotenoids. Moreover, an increase in ROS was also reported followed by up-regulation of ROS-detoxifying enzymes, indicating high oxidative damage. Thus future studies are well-positioned to take the findings of this thesis deeper into identifying the molecular mechanisms that control the underlying physiological pathways in microalgal cells, including the identification of different key genes and proteins that are differentially regulated in response to UV-C induction. This may lead to the development of different strategies to manipulate microalgal cells based on the desired compounds.

Optimisation of UV-C settling technique

While conducting experiments on flagellate *Tetraselmis* sp. it was discovered that UV-C treatment not only induced lipids in these microalgae but also induced settling. But as UV-C settling experiments were conducted alongside with lipid induction studies, only apparent experiments were carried out; hence there was a need to conduct more detailed settling experiments on different flagellate microalgae. Moreover, there was a need to compare UV-C settling experiments other harvesting techniques like sedimentation, flotation and centrifuge.

UV-C for extraction of oil from microalgae

In the UV-C studies conducted on different microalgal species, along with the increase of cell size, the cell survival rate declined dramatically. Moreover, as the cell walls became fragile after higher UV radiation, some of the cells had burst open and lipid bodies were released in the medium, especially for *Chlorella* sp. BR2 and *Tetraselmis* sp. M8. Given the difficulty of breaking cells for lipid extraction, this “adverse impact” can be praised, as both lipid stimulation and extraction could be achieved by proper use of UV-C radiation on microalgae. Thus future research will be conducted into optimisation of the UV-C technique, not only to induce high valve compounds and settling but also as a pretreatment to more efficiently extract the desired compounds.

Optimisation of UV-C method for other microalgal species

Considering that microalgae are one of most varied and diverse groups of organisms that are present on the planet, moreover Australia being blessed with one of the longest coastline and various climate zones, there are tremendous opportunities to discover new strains of microalgae and to optimise the UV-C method for these strains to induce lipids, promote settling and/or improve compound extraction efficiency.

Appendices

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Article

High Lipid Induction in Microalgae for Biodiesel Production

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Abstract: Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. High lipid productivity of dominant, fast-growing algae is a major prerequisite for commercial production of microalgal oil-derived biodiesel. However, under optimal growth conditions, large amounts of algal biomass are produced, but with relatively low lipid contents, while species with high lipid contents are typically slow growing. Major advances in this area can be made through the induction of lipid biosynthesis, e.g., by environmental stresses. Lipids, in the form of triacylglycerides typically provide a storage function in the cell that enables microalgae to endure adverse environmental conditions. Essentially algal biomass and triacylglycerides compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis. There has been a wide range of studies carried out to identify and develop efficient lipid induction techniques in microalgae such as nutrients stress (e.g., nitrogen and/or phosphorus starvation), osmotic stress, radiation, pH, temperature, heavy metals and other chemicals. In addition, several genetic strategies for increased triacylglycerides production and inducibility are currently being developed. In this review, we discuss the potential of lipid induction techniques in microalgae and also their application at commercial scale for the production of biodiesel.

Keywords: algaculture; biofuels; biodiesel; induction; lipids; microalgae; oil production; triacylglycerides

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1. Introduction

Sustainable production of renewable energy is being debated globally since it is increasingly understood that first generation biofuels, primarily produced from food crops and mostly oil seeds, compete for arable land, freshwater or biodiverse natural landscapes and are limited in their ability to achieve targets for biofuel production. These concerns have increased the interest in developing second and third generation biofuels such as lignocellulosics and microalgae, respectively, which potentially offer great opportunities in the longer term and do not need to compete for arable land and precious freshwater [1,2]. Due to continuous and increasing combustion of fossil carbon, the amount of greenhouse gas CO_2 has increased. As a result global warming and climate change are threatening ecological stability, food security and social welfare [3,4]. The transportation and energy sector are the two major sources, responsible for the generation of 20% and 60% of greenhouse gases (GHG) emissions, respectively, and it is expected that with the development of emerging economies the global consumption of energy will rise considerably and this will lead to more environmental damage [5].

Photosynthesis is the only process that can convert CO_2 into organic compounds with high energy content, and thus can provide a source for sustainable transport fuel production. There is an urgent need to develop technologies which are able to produce an additional five to six billion tons of organic carbon apart from the current harvest from agricultural crops [3]. Large-scale cultivation of microalgae may be 10–20 times more productive on a per hectare basis than other biofuel crops, are able to use a wide variety of water sources, and have a strong potential to produce biofuels without the competition for food production [2]. Algae can be produced either as macrophytes via marine aquaculture [6] or in large-scale microalgae cultivation systems in open ponds or in photobioreactors [1]. Microalgae are currently considered the most promising types of algae for biofuel production, based on their high lipid contents. Recent progress in the production of microalgae has been intensively reviewed [7], and future perspectives have been presented by Stephens *et al*. [5]. Microalgae can also be cultivated as an integrated concept with wastewater treatment to optimize the energetic and financial input for the production process [8].

Triacylglycerides (TAGs) generally serve as energy storage in microalgae that, once extracted, can be easily converted into biodiesel through transesterification reactions [3,9]. These neutral lipids bear a common structure of triple esters where usually three long-chain fatty acids (FAs) are coupled to a glycerol molecule. Transesterification displaces glycerol with small alcohols (e.g., methanol). Recently, the rise in petroleum prices and the need to reduce greenhouse gas emission has seen a renewed interest in large-scale biodiesel production [10].

Within the last few decades the concept of lipid induction in microalgae has been intensively studied to increase TAG production in microalgae, but at present different lipid induction techniques have not been compared to each other. Here we provide a review of different lipid inducing techniques in microalgae and their potential to be used for biodiesel production.

2. Lipids in Microalgae

Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. Lipids produced by microalgae can be grouped into two

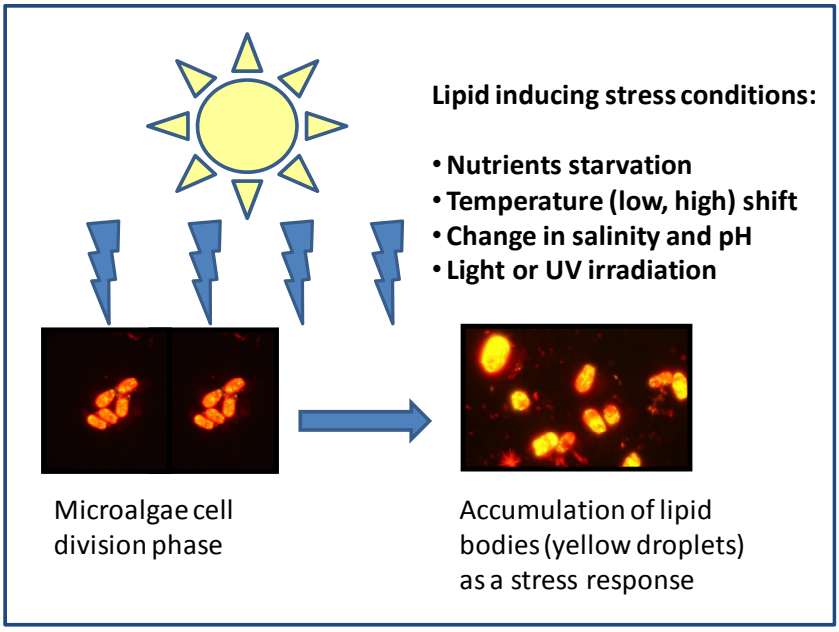
categories, storage lipids (non-polar lipids) and structural lipids (polar lipids). Storage lipids are mainly in the form of TAG made of predominately saturated FAs and some unsaturated FAs which can be transesterified to produce biodiesel. Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs), which are also essential nutrients for aquatic animals and humans. Polar lipids (phospholipids) and sterols are important structural components of cell membranes which act as a selective permeable barrier for cells and organelles. These lipids maintain specific membrane functions, providing the matrix for a wide variety of metabolic processes and participate directly in membrane fusion events. In addition to a structural function, some polar lipids may act as key intermediates (or precursors of intermediates) in cell signaling pathways (e.g., inositol lipids, sphingolipids, oxidative products) and play a role in responding to changes in the environment.

Of the non-polar lipids, TAGs are abundant storage products, which can be easily catabolized to provide metabolic energy [11]. In general, TAGs are mostly synthesized in the light, stored in cytosolic lipid bodies, and then reutilized for polar lipid synthesis in the dark [12]. Microalgal TAGs are generally characterized by both, saturated and monounsaturated FAs. However, some oil-rich species have demonstrated a capacity to accumulate high levels of long-chain polyunsaturated fatty acids (PUFA) as TAG [13,14]. A detailed study on both accumulation of TAG in the green microalga *Parietochloris incisa* and storage into chloroplastic lipids (following recovery from nitrogen starvation) led to the conclusion that TAGs may play an additional role beyond being an energy storage product in this alga [13,15]. Hence, PUFA-rich TAGs are metabolically active and are suggested to act as a reservoir for specific fatty acids. In response to a sudden change in the environmental condition, when the *de novo* synthesis of PUFA may be slower, PUFA-rich TAG may donate specific acyl groups to monogalactosyldiacylglycerol (MGDG) and other polar lipids to enable rapid adaptive membrane reorganization [15,16].

3. Methods of Lipid Induction

The ability of microalgae to survive in diverse and extreme conditions is reflected in the tremendous diversity and sometimes unusual pattern of cellular lipids obtained from these microalgae [17]. Moreover, some of these microalgae can also modify lipid metabolism efficiently in response to changes in environmental conditions [12,18]. A review of microalgal lipid metabolism has recently been published [19]. Under optimal growth conditions, large amounts of algal biomass are produced but with relatively low lipid contents (Figure 1), which constitute about 5–20% of their dry cell weight (DCW), including glycerol-based membrane lipids. Essentially, microalgae biomass and TAGs compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis. Under unfavorable environmental or stress conditions many microalgae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20–50% DCW), mainly in the form of TAG, enabling microalgae to endure these adverse conditions (Figure 1).

Figure 1. Lipid induction in microalgae under stress condition.



High capital costs due to low lipid productivity of FA-synthesizing microalgae are a major bottleneck, hindering the commercial production of microalgal oil-derived biodiesel. One who has grown microalgae under laboratory or outdoor condition is well aware of the fact that to obtain high lipid content, external stress or lipid induction techniques need to be applied. Many microalgae produce saturated and unsaturated FAs naturally under ideal growth conditions, which have high nutritional value, but are less ideal for biofuels. However, the synthesis of neutral lipids in the form of TAG can be induced in many species under stress conditions, and these lipids are suitable precursors for biodiesel production [20,21]. The occurrence and the extent to which TAGs are produced is species/strain-specific, and are ultimately controlled by the genetic make-up of individual organisms. Synthesis and accumulation of large amounts of TAGs accompanied by considerable alterations in lipid and FA composition can occur in microalgae when placed under stress conditions imposed by chemical or physical environmental stimuli, either acting individually or in combination [21]. There has been a wide range of studies carried out on lipid induction techniques in microalgae such as the use of nutrients stress, including nitrogen and/or phosphorus starvation, light irradiation, pH, temperature, heavy metals and other chemicals. The following paragraphs review the different TAG induction techniques and discuss their potential in different microalgae species.

3.1. Nutrient Starvation

Nutrient availability has a significant impact on growth and propagation of microalgae and broad effects on their lipid and FA composition. Environmental stress condition when nutrients are limited, invariably cause a steadily declining cell division rate. Surprisingly, active biosynthesis of fatty acids is maintained in some algae species under such conditions, provided there is enough light and CO_2 available for photosynthesis [12]. When algal growth (as measured by cell divisions) slows down and there is no requirement for the synthesis of new membrane compounds, the cells instead divert and deposit fatty acids into TAG. Under these conditions, TAG production might serve as a protective

mechanism. Under normal growth conditions, ATP and NADPH produced by photosynthesis are consumed by generating biomass, with ADP and $NADP^+$ eventually being available again as acceptor molecules in photosynthesis. When cell growth and proliferation is impaired due to the lack of nutrients, the pool of the major electron acceptor for photosynthesis, $NADP^+$, can become depleted. Since photosynthesis is mainly controlled by the abundance of light, and cannot be shut down completely, this can lead to a potentially dangerous situation for the cell, damaging cell components. NADPH is consumed in FA biosynthesis, therefore, increased FAs production (which in turn are stored in TAGs) replenishes the pool of $NADP^+$ under growth-limiting conditions [12,21].

Nutrient starvation is one of the most widely used and applied lipid induction techniques in microalgal TAG production and has been reported for many species (Table 1). For example, when the diatom *Stephanodiscus minutulus* was grown under silicon, nitrogen or phosphorus limitation, an increase in TAG accumulation and a decrease of polar lipids (as percentage of total lipids) was noticed in all of the nutrient-limited cultures [22]. In the green alga *Chlamydomonas moewusii*, nutrient limitation resulted in decreased PUFA C16:3, C16:4, and C18:3 contents whereas overall levels of C16:1 and C18:1 FA were increased [23].

Nitrogen is the single most critical nutrient affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly TAG, in response to nitrogen deficiency has been observed in numerous species or strains of various microalgae [24–26]. Hu *et al.* [27] conducted a study on nitrogen stress responses of several green microalgae, diatoms and cyanobacteria and all tested species showed a significant rise in lipid production. A detailed and large-scale model of lipid induction by nutrient starvation (nitrogen, phosphorus) on several diatoms, green algae, red algae, prymnesiophytes and eustimatophytes is presented in a study carried out by Rodolfi *et al.* [28]. In the diatom *Cyclotella cryptica,* higher levels of neutral lipids (primarily TAG) and higher proportions of saturated and mono-unsaturated FAs were produced due to silicon deficiency [20]. However, only a small increase in TAG levels (from 69 to 75% from total lipids) together with phospholipids (from 6 to 8%) was reported for the microalga *Phaeodactylum tricornutum* as a result of reduced nitrogen concentrations [29]. *Scenedesmus* sp. subjected to nitrogen or phosphorus limitation showed an increase in lipids as high as 30% and 53%, respectively [30]. Lipid content of freshwater green alga *Chlorella vulgaris* could be significantly increased by 40% in low nitrogen-containing medium [31]. With manipulated culture conditions of 1 mM KNO_3, 1.0% CO_2, 60 μmol photon m^{-2} s^{-1} and 25 °C, lipid production of *C. vulgaris* was increased by 2.5-fold [32]. In addition, lipid stimulation in *Chlorella* was also achieved via silicon deficiency [33] and iron supplementation [34]. Moreover, it was found for *C. vulgaris* that changing from normal nutrient to nitrogen depletion media gradually changed the lipid composition from free FA-rich lipids to lipid mostly contained as TAG [35]. Nitrogen starvation in microalgae not only affects the fatty acid metabolism, but also affects pigment composition. For *Parietochloris incise* grown in nitrogen-replete medium a considerable increase in the ratio of carotenoid and chlorophyll contents was recorded [36].

Phosphorus limitation resulted in increased lipid content, mainly as TAG, in *P. tricornutum*, *Chaetoceros* sp., *Isochrysis galbana* and *Pavlova lutheri*, but decreased lipid content in *Nannochloris atomus* and *Tetraselmis* sp. [37]. Due to phosphorus deprivation, production of C16:0 and C18:1 was increased and production of C18:4ω3, C20:5ω3 and C22:6ω3 was decreased [37]. In contrast, for phosphorus-starved cells of the green alga *Chlorella kessleri*, an elevated level of unsaturated fatty

acids in all identified individual lipids, namely phosphatidylcholine (PC), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), and sulfoquinovosyl diacylglycerols (SQDG) were found [38]. Phosphorus limitation was also found to increase the overall TAG production from 6.5% up to 39.3% with a gradual decrease in eicosapentaenoic acid (EPA) concentration. The cellular total lipid content increased, mainly due to TAG accumulation in *Monodus subterraneus* [15]. In studies carried out on other organisms, including higher plants, the authors have also acknowledged replacement of membrane phospholipids by non-phosphorus containing glycolipids and betain lipids under phosphate limitation [39,40].

A study by Sato *et al.* [17] on sulphur and phosphorus depletion in green alga *C. reinhardtii* showed that sulphur depletion leads to decrease in SQDG but on the other hand PG was increased by 2-fold, representing a compensatory mechanism where lipids containing sulphur are substituted by lipids containing phosphate. When *C. reinhardtii* was grown in media with limited phosphorus it showed a 40% decrease in PG and also stimulated increase in the SQDG content. Thus, mechanisms that keep the total sum of SQDG and PG concentrations constant under both phosphorus and sulphur-limiting conditions appear to occur [17]. Other studies have also shown that sulfur deprivation led to increased total lipid content in the green algae *Chlorella* sp. and *C. reinhardtii* [41].

Based on the literature reviewed, it is clear that amongst all nutrient starvation approaches, nitrogen starvation technique is most widely applied and studied in almost all the microalgae species that can be considered for the production of biofuel (Table 1). Nitrogen is the most growth-limiting factor for eukaryotic microalgae and would be one of the first nutrients to be depleted during algae cultivation. It is relatively easy to apply controlled nitrogen stress on microalgae by subtracting the nitrogen source in the growth media. Moreover all the microalgae species studied so far (Table 1), seem to increase TAG production under nitrogen stress. Hence, nitrogen starvation is the most successful lipid inducing technique at present. However, high lipid production due to nitrogen stress may take 2–5 days and is complemented with slow growth rates and low cell counts and thus finally effecting the total biomass and lipid productivity as detailed by Widjaja *et al.* [35].

Table 1. Examples of different types of nutrient starvation stress which have been studied to induce lipids in microalgae.

Microalgae species or strain	Nutrient stress	Changes in lipid profile after induction	Reference
Chlamydomonas reinhardtii, Scenedesmus subspicatus	Nitrogen limitation	Increase in total lipids (lipid: amide ratio)	[42]
Nannochloropsis oculata	Nitrogen limitation	Total lipid increased by 15.31%	[43]
Chlorella vulgaris	Nitrogen limitation	Total lipid increased by 16.41%	[43]
Chlorella vulgaris	Nitrogen limitation	Lipid productivity of 78 mg/L d	[24]
***Chlorella* sp.**	Nitrogen limitation	Lipid productivity of 53.96 ± 0.63 mg/L d	[25]
Phaeodactylum tricornutum	Nitrogen limitation	TAG levels increased from 69 to 75%	[29]

Table 1. *Cont.*

Dunaliella tertiolecta	Nitrogen limitation	Five times increase in lipid fluorescence	[44]
Chlorella vulgaris	Nitrogen medium	Lipids increased by 40%	[31]
Chlorella vulgaris	Nitrogen limitation	Increase in TAG	[35]
***Chlorella* sp.**	Nutrient-deprived conditions (nitrogen, phosphate-potassium, iron, and all three combined)	Total lipid production of 49.16 ± 1.36 mg/L d	[25]
***Chlorella* sp.**	Urea limitation	Total lipid productivity of 0.124 g/ L d	[26]
Neochloris oleoabundans	Ammonium nitrate	Lipid productivity of 0.133 g /L d	[45]
***Scenedesmus* sp., *Coelastrella* sp.**	Combined effect of Ph and N-limitation	Increase in TAG	[46]
Phaeodactylum tricornutum* , *Chaetoceros* sp., *Isochrysis galbana	Phosphorus limitation	Increase in total lipids with higher relative content of 16:0 and 18:1	[37]
Monodus subterraneus	Phosphorus limitation	Increase in TAG	[15]
***Scenedesmus* sp**	Nitrogen and phosphorus starvation	Lipids increased 30% and 53%, respectively	[30]
***Chlorella* sp.**	Silicon deficiency	-	[33]
Chlorella kessleri	Phosphorus limitation	Increase in unsaturated FAs	[38]
Chlamydomonas reinhardtii	Sulphur limitation	PG was increased by 2-fold	[17]
Chlamydomonas reinhardtii	Sulphur limition	Increase in TAG	[41]
Cyclotella cryptica	Silicon starvation	Increased in total lipids from 27.6% to 54.1%	[47]

3.2. Temperature Stress

Temperature has been found to have a major effect on the fatty acid composition of microalgae (Table 2) [18,48]. A general trend towards increasing FA unsaturation with decreasing temperature and increasing saturated FA with increasing temperature has been observed in many microalgae and cyanobacteria (Table 2) [49–52]. It is generally accepted that many of the lipid profile changes alter the physical properties of membranes so that normal functions (e.g., ion permeability, photosynthetic and respiratory processes) can continue unimpaired [53]. The most commonly observed change in membrane lipids following a temperature shift is an alteration in FA unsaturation [54]. Due to their geometry, FAs with carbon-carbon double bonds cannot be as densely packed as saturated FA, therefore the fluidity of membranes containing unsaturated FA is increased. As membrane fluidity is decreased at lower temperatures, increased FA unsaturation provides an adaptation to the changing environment.

Dunaliella salina has been extensively analyzed for low temperature modification of lipid composition [12]. A temperature shift from 30 °C to 12 °C increased the level of unsaturated lipids significantly by 20% [12]. In *Ochromonas danica*, as the incubation temperature rose from 15 to

30 °C, the cell number per unit volume of medium was increased thus increasing total lipid content [55]. In *Chlorella vulgaris* and *Botryococcus braunii*, increased temperature resulted in a decrease of the relative content of unsaturated intracellular fatty FAs [56]. Increases in growth rate and total lipid production were obtained in *Nannochloropsis salina* with an increase in temperature [57]. Whereas, a decrease in culture temperature from 25 to 10 °C led to an elevation in the relative proportion of oleate but a decrease in linoleate and stearidonic acid (C18:4n-3) in the green alga *Selenastrum capricornutum* [58]. In a culture of *I. galbana* grown at 30 °C, total lipids accumulated at a higher rate with a slight decrease in the proportion of nonpolar lipids [59]. On the other hand, higher levels of omega-3 PUFA α-linolenic acid (ALA) and docosahexaenoic acid (DHA) with a corresponding decrease in saturated, monounsaturated, and linoleic fatty acids were found in the cells grown at 15 °C [59]. Moreover, in the diatom *P. tricornutum* the highest yields of PUFA and EPA per unit dry mass were 4.9 and 2.6%, respectively, when temperature was shifted from 25 °C to 10 °C for 12 h, with both being raised by 120% compared with the control [60].

Study on the effects of low temperatures in some higher plants have also been shown to increase the amount of unsaturated FAs [61]. Similar results were also obtained in *Chlorella ellipsoidea* where the content of unsaturated FA was increased by 2-fold. Moreover, a low temperature-adapted strain of this species also showed increased ALA and, therefore, more unsaturation in its PG [62]. In the marine microalga *Pavlova lutheri*, significant changes in acidic lipid and fatty acid composition have been reported for cultures grown at 15 °C compared with 25 °C [63]. The culture grown at 15 °C resulted in an increased relative amount of EPA and DHA [63]. Variations of FA composition with growth temperature were also studied by Fork *et al.* on the thermophilic cyanobacterium *Synechococcus lividus* [64]. When the growth temperature was lowered from 55 °C to 38 °C, the amount of saturated FA C18:0 decreased while the unsaturated FAs C18:1 and C16:1 increased [64]. In general, there was an increase in the more fluid lipids in all of the lipid classes when the cells were grown at the lower temperature [64].

The cyanobacterium Sp*irulina platensis* and eukaryotic microalgae *Chlorella vulgaris* and *Botryococcus braunii* were studied for the effect of ambient temperature on the composition of intracellular FAs and the release of free fatty acids (FFA) into the medium [56]. It was found that all of the above species studied, regardless of their taxonomic status, responded to the temperature regime by similar changes in their intracellular FA composition: the relative content of more unsaturated FAs decreased and saturated FAs increased with the elevation of temperature [56].

In contrast, no significant change in the lipid content was observed in *Chlorella sorokiniana* grown at various temperatures [65]. There was no effect of temperature shift on the content of the acidic thylakoid lipids, SQDG and PG, in *C. reinhardtii* [17]. It should be noted that only a limited amount of information is available on this subject and that all studies were carried at laboratory scale where it is very easy to maintain the desired temperature. Thus maintaining, decreasing or increasing temperature is feasible only in closed system photobioreactors which are costly when compared to open systems. At present, we are not aware of any study that has highlighted the effect of temperature to induce lipids on large-scale cultivation; but as lipid profiles clearly change at different temperatures, properties of algal-derived biodiesel would also change for different climates and seasons. Different strains or species may be used for different seasons (e.g., summer or winter strains) and efforts are underway to use flue gases and other heat sources to increase algae growth in colder climates.

Table 2. Lipid induction in microalgae with different temperatures.

Microalgae species or strain	Stressing agent	Lipid profile change after induction	Reference
***Chaetoceros* sp.**	Grown at 25 °C	Total lipid increased by 16.8%	[49]
***Rhodomonas* sp., *Cryptomonas* sp., *Isochrysis* sp.**	Range of 27 °C to 30 °C	Lipid production increased by 15.5, 12.7, and 21.7% respectively	[49]
Nannochloropsis oculata	Increase from 20 °C to 25 °C	Lipid production increased by 14.92%	[43]
Isochrysis galbana	Increase from 15 °C to 30 °C	Increase in neutral lipids	[59]
Chlorella ellipsoidea	Lowering temperature	Unsaturated FA was increased by 2-fold	[62]
Nannochloropsis salina	Increase in temperature	Increase in total lipids	[57]
Dunaliella salina	Shift from 30 °C to 12 °C	Increase in unsaturated lipids	[12]
Ochromonas danica	Increase from 15 °C to 30 °C	Increase in total lipids	[55]
Selenastrum capricornutum	Temperature from 25 °C to 10 °C	Increase in oleate fatty acid	[58]
Isochrysis galbana	Grown at 30 °C	Increase in total lipids	[59]
Phaeodactylum tricornutum	Shifted from 25 °C to 10 °C for 12 h	Highest yields of PUFA and EPA	[60]
Pavlova lutheri	Grown at 15 °C	Increased relative amount of EPA	[63]
Spirulina platensis, Chlorella vulgaris, Botryococcus braunii	Increase in temperature	Saturated FAs increased	[56]

3.3. Salinity-Induced Lipid Production

Dunaliella species provide the best examples of microalgae that can tolerate high salt concentrations. The ability of *Dunaliella* species to proliferate over practically the saturation range of salinities makes them one of the favorite candidates to study salinity effects on microalgae [66–68]. In a study carried out by Azachi *et al.* [66] cells of *D. salina* were transferred from 0.5 to 3.5 M (29 to 205 g/L) NaCl, and there was a significantly higher ratio of C18 (mostly unsaturated) to C16 (mostly saturated) FAs in the cells grown in 3.5 M (205 g/L) NaCl compared with those grown at 0.5 M (29 g/L) NaCl [66]. An increase of the initial NaCl concentration from 0.5 M (29 g/L) to 1.0 M (58 g/L) followed by further addition of NaCl to 2.0 M (117 g/L) during cultivation of *Dunaliella tertiolecta* resulted in an increase in intracellular lipid content and a higher percentage of TAG [67]. An even stronger increase in salinity from 0.4 M to 4 M (23 to 234 g/L) in *Dunaliella* sp. increased the proportion of total saturated fatty and monounsaturated fatty acids, whereas the proportion of PUFA was decreased [68].

The diatom *Nitzschia laevis* is known to produce high amounts of EPA [69]. When these cells were subjected to high salt concentrations, the degree of FA unsaturation of both neutral and polar lipid fractions increased sharply when salt concentrations increased from 10 to 20 g/L, but decreased at salt concentrations of 30 g/L [69]. Highest contents of total fatty acids, EPA and polar lipids were all obtained at NaCl concentration of 20 g/L, under which 71.3% of total EPA existed in polar lipid

fractions [69]. The amount of total free sterols was also increased with an increase in salt concentration. In three marine heterotrophic microalgae strains, *Crythecodinium cohnii* ATCC 30556, *C. cohnii* ATCC 50051 and *C. cohnii* RJH grown at different salinities, the FA composition was also affected [70]. At 9 g/L NaCl, *C. cohnii* ATCC 30556 had the highest total FA content and DHA (C22:6) proportion. In contrast, *C. cohnii* ATCC 50051 and *C. cohnii* RJH had the highest DHA content at 5 g/L NaCl. *C. cohnii* ATCC 30556 and ATCC 50051 had the highest DHA yield (132 and 68 mg/L respectively) at 9 g/L NaCl while *C. cohnii* RJH had the highest DHA yield (129 mg/L) at 5 g/L NaCl [70]. Growth, lipid content and FA composition of heterotrophic microalga *Schizochytrium limacinum* OUC88 at different temperatures (16 °C, 23 °C, 30 °C and 37 °C) and salinities (0, 9, 18, 27 and 36 g/L) were analyzed [71]. Highest lipid content was obtained at salinities of 9–36 g/L at a temperature range of 16–30 °C and the content of saturated fatty acids C15:0 and C17:0 was increased greatly [71]. In addition, the ratio of DHA to DPA changed at different temperatures and salinities [71].

3.4. The Effect of pH and Heavy Metals Stress

Fluctuations of the pH in the medium also have been found to alter the lipid composition of microalgae (Table 3). For example, alkaline pH stress led to TAG accumulation in *Chlorella* CHLOR1 and was not dependent on nitrogen or carbon limitation levels, and led to a decrease in membrane lipids [72]. Based on morphological observations, alkaline pH inhibited the growth of microalgae, thus diverting the energy to form TAG [72]. The effects of pH on the lipid and FA composition of a *Chlamydomonas* sp. isolated from a volcanic acidic lake, and *C. reinhardtii* have been studied and compared [73]. In the unidentified *Chlamydomonas* sp., FAs of polar lipids were more saturated than those in *C. reinhardtii*. The relative proportion of TAG (as percentage of total lipids) was higher in *Chlamydomonas* sp. grown at pH 1 than that in the cells cultivated at higher pH. The increase in saturation of fatty acids in membrane lipids of *Chlamydomonas* has been suggested to represent an adaptive reaction at low pH to decrease membrane lipid fluidity [73].

Heavy metals like cadmium, iron, copper and zinc have also been found to increase the lipid content in some microalgae [74]. The effect of high levels of cadmium was studied in *Euglena gracilis* grown as autotrophic, heterotrophic (in the dark) and mixotrophic (in the light with an organic carbon source) cultures [74]. Cadmium caused an increase in the total lipid content per cell in all three culture systems [74]. Among the membrane lipids, sterol content was lower in cadmium-treated cells cultivated under illumination. There were no changes in the total phospholipid content, although there was an increase in PG. *E. gracilis* has also been shown to display somewhat different sensitivities to copper and zinc [74]. The effect of iron on growth and lipid accumulation in *Chlorella vulgaris* was investigated by Liu *et al.* [34]. The culture in the late exponential growth phase when supplemented with Fe^{3+} at different concentrations, showed increased total lipid content of up to 56.6% biomass by dry weight [34].

Table 3. Examples of lipid induction in microalgae due to salinity and pH stress.

Microalgae sp	Salinity change	Lipid profile change after induction	Reference
Dunaliella salina	Transferred from 29 to 205 g/L NaCl	Increased concentration of C18 FA	[66]
Dunaliella tertiolecta	Transferred from 29 g/L to 58 g/L NaCl	Increase in lipid content and TAG	[67]
***Dunaliella* sp.**	Increased salinity from 23 to 234 g/L NaCl	Increase in total FA and monounsaturated FA	[68]
Nitzschia laevis	NaCl concentration increased from 10 to 20 g/L	Increase in unsaturated FA	[69]
***Crythecodinium. cohnii* ATCC 30556**	At 9 g/L NaCl	Increase in total FA content and DHA	[70]
Schizochytrium limacinum	Salinity at 9–36 g/L at temperature range of 16–30 °C	Saturated FA C15:0 and C17:0 was greatly increased	[71]
***Unidentified Chlamydomonas* sp.**	Low pH	Increase in saturated FAs	[73]
***Chlorella* sp.**	alkaline pH	Increase in TAG	[72]
Euglenia gracilis	Cadmium, copper, zinc	Increase in total lipids	[74]
Chlorella vulgaris	Fe^{3+}	Increase in total lipids to 56.6% of biomass	[34]

3.5. Light Irradiation Stress

Light is the most important element for photosynthesis, without which no autotrophic life can sustain or flourish. Microalgae have been reported to grow on various light intensities exhibiting remarkable changes in their gross chemical composition, pigment content and photosynthetic activity [75] (Table 4). Moreover, different light intensities and wavelengths have been reported to change the lipid metabolism in microalgae altering the lipid profile [76] (Table 4). High light intensity leads to oxidative damage of PUFA [76], and is also required for the synthesis of C16:1 (3 *trans*) and alters the level of this fatty acid in microalgae. Typically, low light intensity induces the formation of polar lipids, particularly the membrane polar lipids associated with the chloroplast, whereas high light intensity decreases total polar lipid content with a simultaneous increase in the amount of neutral storage lipids, mainly TAGs [77–80]. High light exposure decreased the total phospholipid content and increased the level of nonpolar lipid (namely TAG) in the filamentous green alga *Cladophora* sp. [79]. In the red microalga *Tichocarpus crinitus* exposure to low light intensity resulted in increased levels of some cell membrane lipids, especially SQDG, PG and PC, whereas higher light intensities increased the level of TAG [78]. In the haptophyte *Pavlova lutheri* higher light intensities act as a catalyst to increase the lipid content and were associated with lower dilution rate-promoted increases in both cell population and weight per cell [81]. TAG production under high light conditions might serve as a protective mechanism for the cell. As outlined above, electron acceptors needed by the photosynthetic machinery might be depleted under high light conditions as well. Increased FA synthesis which in turn are stored as TAG, potentially helps the cell to re-generate its electron acceptor pool.

Light intensity not only affects the fatty acid composition in microalgae, but also the pigment composition. In the green microalga *Parietochloris incise* under low irradiance photosynthetically active radiation, cultures displayed slow growth and a relatively low carotenoid-to-chlorophyll ratio [36]. At higher irradiances on complete medium, the alga displayed a higher growth rate and an increase in the carotenoid content, especially that of β-carotene and lutein [36].

Light/dark cycles at different growth phases also have a significant effect on algal lipid composition, as was successfully demonstrated in a detailed study on various light regimes on lipids of the diatom *Thalassiosira pseudonana* [77]. A culture grown to stationary phase under strong continuous light or under 12:12 h strong light/dark conditions had a higher amount of TAG with saturated and monounsaturated fatty acids compared to cultures grown with less light. At the exponential growth phase, however, the proportion of PUFA was highest under high light conditions [77]. This demonstrates the important role of growth phase in the accumulation of certain fatty acids. With the onset of stationary phase, algae typically show increased proportions of saturated and monounsaturated fatty acids and decreased amounts of PUFA [77]. The lipid and fatty acid compositions of three species of sea ice diatoms grown in chemostats have been analyzed and compared when cultivated at light-limiting conditions of 2 and 15 μmol photons m^{-2} s^{-1} [82]. Growing cultures at 2 μmol photons m^{-2} s^{-1} resulted in 50% more MGDG containing EPA than those grown at 15 μmol photons m^{-2} s^{-1}. Growing cultures at 2 μmol photons m^{-2} s^{-1} resulted in higher amounts of non-polar lipid bilayer-forming MGDG in relation to total bilayer-forming lipids, especially DGDG (the ratio of MGDG:DGDG increased from 3.4 to 5.7) than in cultures grown at 15 μmol photons m^{-2} s^{-1}. A shorter light period seemed to increase the production of PUFA in *Isochrysis galbana* [83]. Sitosterol and stigmasterol were the two main sterols detected at 246.3 and 220.0 mg/100 g, respectively. A continuous increase in the level of total sterols was recorded during the life cycle at 24 h lighting [83]. The reduction of the photoperiod led to a decrease in the total sterols produced in the decay phase. A gradual increase in α-tocopherol production during the life cycle was also recorded [83]. Dark treatment caused a decrease in the relative proportion of oleate fatty acid and an increase in linoleate fatty acid in the green alga *Selenastrum capricornutum* [58]. In the dinoflagellate *Prorocentrum minimum* dark exposure led to a reduced content of TAG and galactosylglycerides, while the total content of phospholipids changed little [58].

Light irradiation can only be controlled in a closed system bioreactors or in laboratory-scale cultures, as shown by the examples above. Moreover, operational costs for controlled light add up to the production cost of biofuels from microalgae, although there are several commercial approaches of using LEDs or diverted sunlight in large-scale photobioreactors. Light is essential for TAG production, but if high light irradiation is used as a stimulant for increased TAG production, based on the examples above and in Table 4, it can be expected that this will differ for different species. In addition, TAG FA composition is different for different species in response to different light exposures. For example, in *Nannochloropsis* sp. the degree of unsaturation of FAs was lower with increasing irradiance with a significant decrease in omega-3 fatty acids (29% to 8% of total FA), caused mainly by a decrease of EPA (20:5n-3) [84]. In conclusion, light will normally stimulate fatty acid synthesis, growth and the formation of (particularly chloroplast) membranes. Therefore, the overall lipid content of algae will reflect such morphological changes.

3.6. UV Irradiance for Lipid Induction

Current research on the effect of UV irradiance in microalgae is mainly focused on the impact of UV-A and UV-B radiation on algal growth, morphology, physiology and oxidative stress [85–89], with a special emphasis on pigments and photosynthesis [90]. Examples of studies on UV radiation on lipid profiles in microalgae are shown in Table 4.

In a study carried out by Srinivas and Ochs [91] on *Nannochloropsis oculata* the effect of UV-A at different levels of exposure on total lipid accumulation was investigated. UV-A treatments significantly increased the PUFA (chlorophyll-specific lipid concentration) of *N. oculata* cells, and UV-A and decreased nutrients had a synergistic effect on lipid accumulation. The effects of UV-B radiation on the total lipid, FA and sterol composition and content of three Antarctic marine phytoplankton species *Odontella weissflogii*, *Chaetoceros simplex* and the *haptophyte Phaeocystis antarctica* were examined in a preliminary culture experiment [92]. The cultures were exposed to constant UV-A and low or high UV-B radiation. The sterol, fatty acid and total lipid content for *Odontella weissflogii* changed little under low UV-B when compared with control conditions. In contrast, when *P. antarctica* was exposed to low UV-B irradiance, storage lipids were reduced and structural lipids increased [92]. *P. antarctica* also contained a higher proportion of polyunsaturated fatty acids under low UV-B exposure. Exposure of *P. antarctica* to high UV-B irradiance increased total lipid, TAG and FFA concentrations. Lipid concentrations per cell also increased when *C. simplex* was exposed to high UV-B irradiance [92]. This resulted from increases in FFA concentration principally saturated FA and may indicate degradation of complex lipids during high UV-B treatment [92]. Effect of UV-B radiation on lipid productivity was studied in detail in *Tetraselmis* sp. [93]. A 4 hour-exposure to UV-B radiation resulted in an overall increase in saturated FA and monounsaturated FA, whereas the PUFA content was decreased by 50% [93]. In addition, UV irradiance caused a decline in the overall rate of carbon incorporated into amino acids and a reduction in the pool size of total cellular amino acids [93]. In contrast, intracellular dissolved free amino acid increased [93].

The effect of UV radiation on growth and fatty acid composition of two diatoms, *P. tricornutum* and *Chaetoceros muelleri*, were examined in batch cultures [94]. UV radiation induced significant differences in all the major fatty acids of *P. tricornutum*. The percentages of EPA and PUFA increased while monounsaturated FA decreased in the UV-A treatment in comparison with no UV irradiance or combined UV-A + UV-B treatments [94]. On the other hand, all the major fatty acids of *C. muelleri* varied with harvest stage and UV irradiance. The percentage of monounsaturated FA in *C. muelleri* increased, while EPA and PUFA decreased under combined UV-A + UV-B treatment [94]. The study indicated that UV-A exposure may promote EPA and PUFA formation in *P. tricornutum*, whereas combined UV-A + UV-B exposure enhanced short FA and monounsaturated FA content, but suppressed PUFA formation in *C. muelleri* [94].

PUFAs, especially EPA and DHA, are abundantly synthesized by some phytoplankton species and play a key role in the marine food chain. However, they are generally considered to be sensitive to oxidation by UV radiation. *P. lutheri* and *Odontella aurita* were exposed to a combination of UV-A and UV-B radiation with a total daily dose of 110 kJ/m^2 and lipid composition was then determined on days 3, 5, and 8 of UV exposure [95]. In *P. lutheri* exposure to UV led to a decrease in the proportion

of PUFAs, especially those in structural lipids (glycolipids and phospholipids) and a reduction of 20% in EPA levels and 16% in DHA levels, after 8 days; whereas for *O. aurita*, exposure to UV did not change the fatty acid composition of the total lipids and lipid fractions of the cells [95].

Interestingly, UV radiation has been suggested for microalgal lipid induction in large-scale cultivation systems. As UV radiation has genetically and physiologically deleterious effects on many life forms including microalgae [95], the impact is conceivably related to the radiation intensity. A recent study showed that the modulated use of UV-A radiation for seven days could lead to an increased production of fatty acids in *Nannocloropsis* sp. [96]. However, there is concern that constant use of UV-A light may not be viable for industrial-scale cultivation, while shorter, but stronger UV radiation could also affect microalgal lipid composition and production as a result of inhibiting nutrient uptake, carbon assimilation mechanism and damaging DNA [97].

Table 4. Lipid induction due to different light irradiation stress in microalgae.

Microalgae sp	Irradiation type	Lipid profile change after induction	Reference
Tichocarpus crinitus	Low light intensity	Increased TAG	[78]
Pavlova lutheri	High light intensities	Increased total lipid content	[81]
Thalassiosira pseudonana	Continuous or light/dark cycled strong light at exponential growth	Increased PUFA	[77]
Thalassiosira pseudonana	Continuous or light/dark cycled strong light at stationary phase	Increased TAG	[77]
Unidentified diatoms	Low light (2 μmol photons m^{-2} s^{-1})	50% more MGDG	[82]
Selenastrum capricornutum	Dark treatment	Increase in linoleate FA	[58]
Prorocentrum minimum	Dark treatment	Marginal increase in phospholipids	[58]
Isochrysis galbana	Shorter light period	Increase of PUFA	[83]
Nannochloropsis oculata	UV-A	Increase of PUFA, structural lipids	[91]
P. antarctica	Low UV-B	Increase in PUFA, structural lipids	[70]
C. simplex	High UV-B	Increase in total lipids	[70]
***Tetraselmis* sp.**	UV-B radiation	Increase in saturated and monounsaturated FA	[93]
Phaeodactylum tricornutum	UV radiation	Increased EPA and PUFA	[98]
Chaetoceros muelleri	UV-A	Increased monounsaturated FA	[98]
***Nannochloropsis* sp.**	UV-A	Increase in saturated FA to PUFA ratio	[96]

4. Genetic Engineering of Microalgae to Increase Lipid Production

Apart from inducing lipid biosynthesis in microalgae by external cues, some progress is emerging towards metabolic engineering towards higher TAG or omega-3 accumulation capacities. The model species *C. reinhardtii* has been the focus of most molecular, genetic and physiological

research [99–106]. Significant advances in microalgae genomics have been achieved during the last decade [103,104,106]. Expressed sequence tag (EST) databases have been established; transcriptomes as well as nuclear, mitochondrial and chloroplastidial genomes from several microalgae have been sequenced; and several more are in progress of being sequenced [102]. Of particular relevance in relation to fatty acid biosynthesis, is acetyl-CoA carboxylase (ACC) which was first isolated from the microalga *Cyclotella cryptica* in 1990 by Roessler [107] and was later successfully transformed into the diatoms *C. cryptica* and *Navicula saprophila* [108]. *ACC1* was over-expressed leading to 2-3-fold enhanced enzyme activity. These experiments demonstrated that *ACC* could be transformed efficiently into microalgae although no significant increase of lipid accumulation was observed in the transgenic diatoms [108]. It also suggests that over-expression of ACC enzyme alone might not be sufficient to significantly enhance the lipid biosynthesis pathway. For a recent review on the potential of metabolic engineering and other genetic targets to enhance lipid accumulation in microalgae, see Schuhmann *et al.* [19]. However, it should be pointed out that at present, GM strains of microalgae can only be used in small-scale closed bioreactors and are very strictly regulated. This may increase the total cost of production when compared to non-GM algae in open pond systems.

5. Conclusions and Future Directions

We have discussed the different lipid induction techniques that can be used to stimulate lipid biosynthesis, in microalgae, in particular TAG. It is clear that different microalgae species react to different stresses by producing different fatty acids or by altering their composition of fatty acids. Thus which techniques to apply for the lipid induction in particular microalgae might depend on the environmental conditions and cultivation systems. Perhaps the most obvious way to advance our understanding of how the environment can alter lipid metabolism in microalgae is to study one species under controlled laboratory conditions. Based on the literature reviewed, it is clear that amongst different lipid induction techniques, nitrogen starvation is most widely applied and studied in almost all the microalgae species that can be considered for the commercial production of biodiesel (Table 1). Change in temperature, pH, salinity and heavy metals can also induce lipids effectively, but may be difficult to regulate on a large-scale cultivation system (Tables 2–4). Rapid induction of lipid biosynthesis maybe achieved through hybrid systems where a nutrient-rich flow culture in exponential phase continuously produces algal biomass of which batches (e.g., 50%) can be transferred to low nutrient conditions but with sufficient light irradiance for photosynthesis. The lipid accumulation phase maybe further shortened by applying a combination of different induction types. For example, this may include a sudden induction, as possible in a hybrid system, combined with nutrient depletion, a salinity/pH change as well as exposure to UV irradiance. The exact combination of induction stresses that provides optimum lipid productivity in a large-scale commercial cultivation system for biodiesel production, will differ for every microalgae strain and depends on nutrient supply, environmental and climatic conditions.

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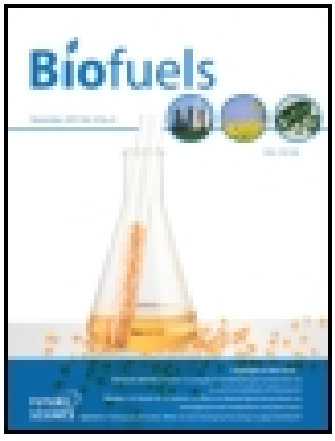

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Critical analysis of current Microalgae dewatering techniques

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Critical analysis of current microalgae dewatering techniques

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Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. However, microalgae harvesting/dewatering is a major obstruction to industrial-scale processing for biofuel production. The dilute nature of microalgae in cultivation creates high operational costs for harvesting, thus making microalgal fuel less economical. Within the last decade, significant advances have been made to develop new technologies for dewatering or harvesting of microalgae. The choice of which harvesting technique to apply depends on the microalgae cell size and the desired product. Microalgae dewatering processes can broadly be classified as primary and secondary dewatering. This article provides an overview of current dewatering techniques along with a critical analysis of costs and efficiencies, and provides recommendations towards cost-effective dewatering.

Microalgae have a robust photosynthetic capability for fixing CO_2 and converting solar energy into chemical energy. Moreover, they do not need to compete with arable land and freshwater, and have been considered as one of the most promising feedstocks for biofuels [1,2]. Microalgae are typically 2–50 µm in size with a negative charge on the cell surface [3–5], but some microalgae, under certain conditions, have a larger cell size. In most cases they are motile (i.e., swimming or gliding), such as dinoflagellates or raphid diatoms, and form stable suspensions. Unfortunately, microalgal biomass is fairly dilute in cultures (up to 0.3–0.5 g dry biomass/l), resulting in difficulties in harvesting and dewatering algae cost effectively [6]. Microalgae harvesting can typically make up to 20–30% of the total biomass production cost [7–9]. This makes the harvesting process a major bottleneck, hindering the development of the microalgae industry. To date, there are a multitude of techniques being used for microalgae dewatering, but with low economical feasibility. Based on their large biodiversity, microalgae harvesting processes are to a large extent species specific [10,11]. They are also closely linked to cell density and cultivation conditions [12].

The production of biofuel, such as biodiesel, from microalgae is a multistep process involving cultivation, biomass harvest, lipid extraction and oil conversion. Compared with the other processes, harvesting is arguably still the most critical and challenging stage in microalgae biomass production [4,8, 12–15]. When considering commercial-scale processes for dewatering and recovering algal biomass for further downstream processes, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting, and the second is known as **secondary dewatering**/thickening **(Figure 1)** [8–10,16]. During the primary harvesting process, the microalgae mass ratio to water volume is increased [17]. This step aims to achieve a concentration containing 2–7% total solid matter, from the initial biomass concentration [16]. Secondary dewatering concentrates the biomass up to 15–25%, which when followed by drying, aims to further concentrate the slurry, increasing the total solid matter up to 90–95%. This step is generally a more energy-intensive step than

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Key terms

Microalgae: Generally unicellular photosynthetic microorganisms, some of which can also form a chain or colony ranging from a few micrometers to a few hundred micrometers.

Secondary dewatering: includes drying. Aims to further concentrate the slurry, increasing the total solid matter up to 90–95%.

Flocculation: Process of forming algae flocs that is often performed as a pretreatment to destabilize algae cells from water and to increase the particle size before using another method such as settling or flotation.

Flotation: Microalgae cells are trapped on micro-air bubbles and float at the surface of water.

Primary dewatering: Aims to concentrate an algae culture from the initial biomass concentration.

primary harvesting. Several techniques for dewatering of microalgae cultures have been developed [16].

This article attempts to provide an overview of these techniques to estimate their efficiencies, and then classify these techniques based on their properties. It also highlights the need for developing hybrid technology. It is desired to optimize microalgae dewatering processes by combining the strengths of several different harvesting techniques.

Primary harvesting

Primary harvesting methods reviewed here include **flocculation**, **flotation**, sedimentation and electroflocculation **(Figure 1)** [1,6,18].

▪ Flocculation

Flocculation is often performed as a pretreatment to increase the particle size before using another method **(Table 1)**. Hence, flocculation is commonly used before secondary dewatering processes to facilitate further steps such as centrifugation or filtration [4,10,19]. In some cases negative charges of microalgae cells inhibit aggregation; therefore, cationic flocculants, cationic polymers and metal salts (e.g., ferric chloride, alum, aluminum sulfate and ferric sulfate) are used to neutralize charges and facilitate aggregation [4,13,16,19–22]. The efficiency of electrolytes to induce coagulation is measured by the critical coagulation concentration, or the concentration required to cause rapid coagulation. Coagulation efficiency of metal ions increases with increasing ionic charge. Multivalent metal salts such as alum have been widely used to flocculate algal biomass in wastewater treatment processes [23,24]. Alum is an effective flocculant for freshwater species such as *Scenedesmus* and *Chlorella* [25]; however, for maximizing the economic value derived from the feedstock, there is a need to produce various co-products such as pigments, protein, omega-3 fatty acids and animal feed along with biofuel production [26]. Hence, flocculation by metal salts may be unacceptable if biomass is to be used in certain aquaculture applications or to be used as food or feed. Polyferric sulphates are reported to be a better flocculant compared with the more traditional nonpolymerized metal salt flocculants, as shown by Jiang *et al.* [27]. Prepolymerized metal salts are effective over a wider pH range than nonpolymerized salts [27]. Moreover, flocculation was carried out by adjustment of pH using sodium hydroxide and addition of the nonanionic polymer Magnafloc LT-25 to a final concentration of 0.5 mgl^{-1} by Knuckey *et al.* [28].

Ultrasound has also been used to induce aggregation in microalgae [29]. Microbial flocculation under nutrient-depletion stress has been investigated by Lee *et al.* [30]. Flocculation occurs naturally in some microalgae; for example, by high light, nitrogen stress, and changes in pH, salinity or the level of dissolved oxygen [22]. This typically leads to flocculation and settling, and probably presents a protective survival mechanism for algae in their natural environment.

Electrolytes and synthetic polymers are typically added to coagulate (neutralize charge) and flocculate the cells, respectively [31]. Smith and Davis recently investigated autoflocculation using magnesium-based flocculants naturally available in brackish water [32]. Moreover, magnesium-based flocculants can be obtained from wastewater treatment plants. A recent study carried out by Taylor *et al.* on *Nannochloropsis oculata* observed that artificially treating the algae with algal extracts does not only effectively flocculate microalgae, but also increase overall lipid content [33]. Interrupting CO_2 to algae culture may also cause autoflocculation [34,35]. However, autoflocculation may not be as reliable as chemical flocculation [22]. Electrocoagulation flocculation (ECF) has been evaluated as a method for harvesting a freshwater (*Chlorella vulgaris*) and a marine (*Phaeodactylum tricornutum*) microalgal species by Vandamme *et al.* [36]. In this study, ECF was shown to be more efficient using an aluminium anode than using an iron anode. Moreover, the efficiency of the ECF process could be substantially improved by reducing the initial pH and by increasing the turbulence in the microalgal suspension. In another study conducted by Xu *et al.*, a rapid and efficient electroflocculation method integrated with dispersed air flotation was developed for harvesting *Botryococcus braunii* with a recovery of 98.6% within 14 min [37].

When considering downstream processes to produce bioproducts from algae, the use of metal salts for coagulation and flocculation poses many challenges. In wastewater sludge treatment, aluminum and sulfate have been shown to affect the specific methanogenic activity of methanogenic and acetogenic bacteria, and reduce their anaerobic digestion ability [38]. A similar problem may be faced when using algal biomass for anaerobic digestion. Land application of aluminum-treated sludge can increase heavy metal uptake and cause phosphorus deficiency in plants [39].

Natural polymers that do not raise environmental concerns may also be used as flocculants, although these are less studied. One of the most widely used and studied natural polymers for flocculation is chitosan (at a pH of ~7), which is typically derived from crab shell. Divakaran *et al.* reported successful flocculation and settling of algae by adding chitosan [40], which is considered an environmentally friendly option that has

Table 1. Examples of various flocculation studies that have been used to harvest microalgae.

Algae	Flocculant	Source	Ph	Dosage	Result	Ref.
Tetraselmis suecica	Praestol	Acrylamide (nonbiological)	8.2	1 mgl^{-1}	70% in 30 min	[47]
Spirulina platensis	Praestol	Acrylamide (nonbiological)	9.4	1 mgl^{-1}	70% in 30 min	[47]
Rhodopseudomonas palustris	Praestol	Acrylamide (nonbiological)	6.8	1 mgl^{-1}	86% in 30 min	[47]
Chaetoceros calcitrans	Magnafloc® LT25	Polyacrylamide (nonbiological)	10.2	>1 mgl^{-1}	93% in 4 h	[48]
C. calcitrans	Chitosan	Inorganic polymer (biological)	8.0	20 mgl^{-1}	83% in 4 h	[48]
Cholrella minutissima	Aluminium chloride	Inorganic salt (nonbiological)	–	0.5 mgl^{-1}	90% in 5 h	[49]
Scenedesmus	Greenfloc 120	Cationic starch	–	>10 mgl^{-1}	<90% in 30 + 30 min	[45]
Phaeodactylum tricornutum	Sodium hydroxide	Alkaline agent	9.8–0.61	–	90–97% in 1 h	[50]
P. tricornutum	Chitosan	Inorganic polymer (biological)	9.9	20 mgl^{-1}	90% in 30 min	[50]
Chlorella vulgaris	Magnesium + sodium hydroxide	–	10.5	0.15 mM	<90% in 30 + 30 min	[51]

also been used in various other studies [41–43]. Other nonconventional flocculants such as *Moringa oleifera* seed flour has been used by Teixeira *et al.* as another nontoxic microalgae flocculant [44]. Cationic starch is also mentioned as another potential effective flocculant for freshwater microalgae by Vandamme *et al.* [45].

▪ Gravity-assisted sedimentation

This process is commonly used in wastewater treatment. However, this process is also appropriate for microalgae larger than 70 µm in size [16,46], but is typically fairly slow due to the low specific gravity of algal cells [4].

▪ Flotation

In this process, microalgal cells are trapped on microair bubbles and float to the surface [16]. Efficient flotation relies on successful collision and attachment of bubbles and particles, and works best when algal cells are hydrophobic [3,47].

Dissolved air flotation (DAF) has been successfully used in water treatment plants and is also widely used for microalgae harvesting (Table 2) [48,49]. It involves the release of pressurized water (saturated with air) into the tank containing microalgae. Due to the difference of pressure, many fine bubbles form, carrying algal cells as a froth, which can be skimmed off. The effectiveness of this process depends on air bubble size, solubility and the pressure difference of air, the hydraulic retention time, and the floated particle size [50]. Before algae can be removed using DAF they need to be flocculated. The flocculation increases the efficiency of removal. A study carried out by Edzwald found DAF to be more effective than sedimentation [50]. Suspended air flotation is an alternative method that could potentially harvest microalgae with a lower air:solids ratio, lower energy requirements and higher loading rates compared with DAF [51].

In dispersed air flotation (or foam flotation), algal cells are floated in a mechanical cell with a high-speed agitator through which a constant stream of air is passed [3]. Fine bubbles of approximately 1 mm diameter are generated by either 'agitation combined with air injection' or 'bubbling air through porous media' [52]. Hydrophobic interaction plays an important role for attachment particles, such as microalgae, to the bubbles [3]. Bubbles then rise to the surface and constantly accumulate as foam as a result of solid–liquid separation [3]. Foam fractionation is considered as an alternative to the use of expensive centrifugation for microalgae harvesting [53].

Secondary dewatering

In secondary dewatering or thickening, the algae slurry is concentrated approximately 10–30-times, and consequently the water content of the produced algae paste can be as low as 20–25% (Figure 1) [13]. Energy-intensive processes such as centrifugation and ultrasonic aggregation are commonly used at this stage [16]. This step requires more energy input than primary dewatering, and therefore needs more capital and operating costs [46].

▪ Centrifugation

Centrifugation is the ideal method for rapid harvesting of algae containing high-value products. Generally centrifuges can be of various types and sizes depending on the uses. A disc stack centrifuge consists of a relatively shallow cylindrical bowl containing a number (stack) of closely spaced metal cones (discs) that rotate, and it is mostly used in commercial plants for high-value algal products and in algal biofuel pilot plants. Decanter centrifuges have been found to be as effective as solid-bowl centrifuges for separating microalgae, but the energy consumption of decanter centrifuges is higher than that of disc-bowl centrifuges at 8 kWh m^{-3} [8]. A hydrocyclone is a relatively low-energy (0.3 kWh m^{-3}) particle-sorting device compared with other centrifuge methods, but on the other hand it was reported

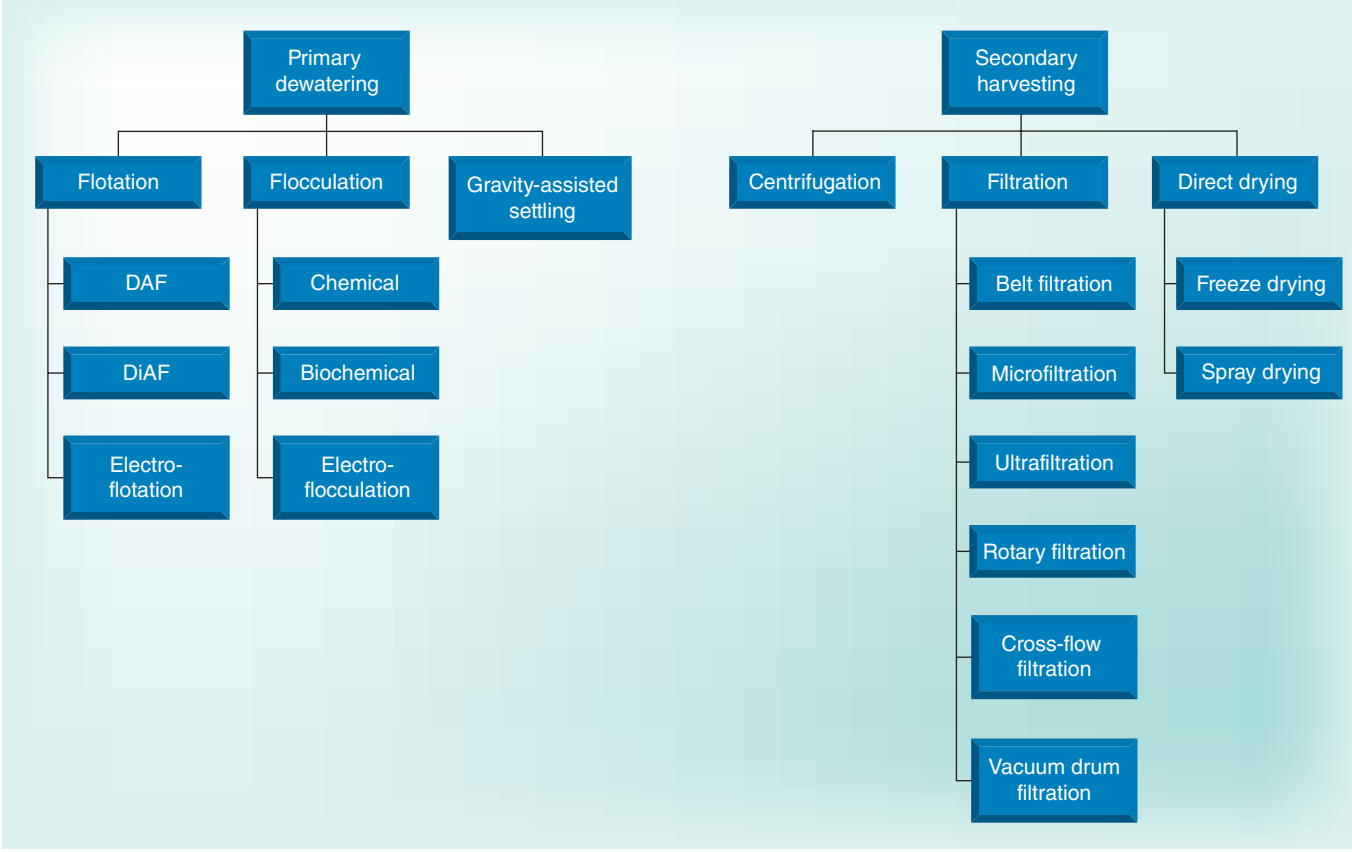

Figure 1. Overview of microalgae harvesting techniques.

to be an unreliable means of concentrating microalgae as only a maximum concentration factor of 4 could be achieved [8]. Spiral plate centrifuges are considered a relatively new generation of centrifuges, manufactured by Evodus. The suspension flows outwards in thin films over vertical plates with the solid sediment or microalgae being forced by centrifugal force to collect on the outer bottom edge of the vanes. Table 3 provides more analyses and details about the harvesting of 10,000 l of *Chlorella* sp. with an Evodos centrifuge. More detailed studies on centrifuge harvesting have been carried out by Molina Grima *et al.* [8]. However, centrifugation is energy intensive, not easily scalable and requires high maintenance due to fast-moving mechanical parts [8,16,22]. Therefore, centrifugation has high capital and operating costs, and is considered too expensive for low-value products such as biofuel [8,54]. Furthermore, high speed spinning can disrupt algae cells [19,55].

Table 2. Examples of various flotation studies that have been used to harvest microalgae.

Algae	Surfactant	Surfactant type	Ph	Dosage (mgl^{-1})	Result	Ref.
Scenedesmus quadricauda	SDS + chitosan	Anionic surfactant	8.0– 5.0	20 + 10	95% in 20 min	[82]
Chlorella sp.	CTAB	Cationic surfactant	8.0	40	86% in 20 min	[83]
Chlorella sp.	SDS + chitosan	Anionic surfactant	8.0–5.0	20 + 10	85–90% in 20 min	[83]
Scenedesmus quadricauda	CTAB	Cationic surfactant	7.8	100	>90% in 20 min	[84]
Chlorella sp.	CTAB	Cationic surfactant	9.5	1–3	95–99% in 11 min	[3]
Chlorella sp.	CTAB	Cationic surfactant		10	45 min	[85]
Dunaliella salina	Aluminium sulphate Ferric sulphate Ferric chloride	Inorganic metallic coagulants	5 5 5	150 150 75	95% in 30 min 98% in 30 min 98.7% in 30 min	[86]

CTAB: Cetyl trimethyl ammonium bromide; SDS: Sodium dodecyl sulfate.

Table 3. Cost of harvesting 10,000 l of *Chlorella* sp. with different harvesting techniques.

	Single step	Primary			Secondary	
	Centrifugation	Sedimentation	Flotation (1)	Flotation (2)	Filtration	Centrifugation
Total energy consumed (kWh/10 m^3)	55[†]	–	7.4–8.4[‡]	0.150[§]	1–3 [4]	5.5[†]
Energy cost (AU$)[¶]	12.10[#]	–	1.62–1.84[#]	0.033	0.22–0.66[#]	1.21[#]
Dosage required (g)	–	100 @ 10 mgl^{-1} [1]	30 @ 3 mgl^{-1} [3]	100 @ 10 mgl^{-1}	–	–
Chemical cost (AU$)	–	2.50 (chitosan @ $25/kg)	0.24 (CTAB @ $8/kg)	0.8 (CTAB @ $8/kg)	–	–
pH adjustment dosage	–	1.5–2 l acetic acid[††]	–	–	–	–
pH adjustment cost (AU$)	–	1.20–1.60 @ $800/ton	–	–	–	–
Total cost (AU$)	12.10	3.70–4.10	1.86–2.08	0.833	0.22–0.66	1.21

[†]An Evodos centrifuge was used for this study.
[‡]Flotation cell considered is Jameson cell and energy consumption was determined using various published studies as well as our own published [3] and unpublished data.
[§]Flotation cell considered is column flotation cell and energy consumption was determined using work done by Coward *et al.* [85].
[¶]1 AU$ = ~US$1.04.
[#]Electricity prices were calculated based on AU$0.22/kWh.
[††]The volume was estimated by doing an experiment with 1 l of algae culture.
CTAB: Cetyl trimethyl ammonium bromide.

▪ Filtration

Filtration methods such as microstrainers, vibrating screen filters, and micro- and ultra-filtration have been widely studied, and have proven to be efficient **(Table 4)** [56–58]. One of the major disadvantages of these techniques is the high capital and operating costs to avoid filter blinding and disruptive pressure changes (high pressure or vacuum). Membrane filtration and ultrafiltration are costly for large-scale operations due to high operating costs for membrane replacement, clogging and pumping [4,8,10,13,16,22,46,57]. Although the filtration process may be considered slower than centrifugation for some applications [10], it is still a simpler and lower cost alternative when compared with centrifugation, if implemented properly. Fast formation of thick filter cake, which dramatically decreases flow rate, is another disadvantage of conventional filtration processes [22].

Cross-flow filtration (tangential flow filtration) has been shown to solve these problems as the filter cake is washed away during the cross-flow filtration process, which increases the operation time of the filtration system [59]. However, this technology is still very expensive for low-value products and is not easily scalable. In addition, most studies consider the conventional filtration process as unsuitable for harvesting of small microalgae (smaller than 30 µm) [4,10,13,16,22,60].

▪ Drying

The water content of algal paste after secondary dewatering should not exceed 50% before oil extraction [12]. Because the cost of thermal drying is high (even higher than mechanical drying), a harvesting method with a high solid content is preferable before drying [10]. Common methods for drying microalgae after secondary dewatering are: spray drying, drum drying, freeze drying and sun drying [10]. Spray drying is considered too expensive for low-value products such as biofuel [10]. The influence of short-term storage and spray and freeze drying of fresh microalgal paste on the stability of lipids and carotenoids of *P. tricornutum* was investigated by Ryckebosch *et al.* [61]. Solar drying is considered the most economical drying process; however, it requires large land areas for large-scale operations [12,62].

Technoeconomic assessment

Using the information from previously completed studies and specifications provided by companies that supply the equipment and chemicals, a theoretical calculation

Table 4. Examples of various filtration studies that have been used to harvest microalgae.

Species	Type of filtration	Effective (% TSS)	Ref.
Coelastrum sp.	Non-precoated vacuum drum filter	18	[6]
Coelastrum sp. and *Scenedesmus* sp.	Potato starch vacuum drum filter	37	[6]
Coelastrum sp.	Belt filter	9.5	[6]
Chlorella and *Cyclotella*	Microfiltration	–	[50]
Scenedesmus quadricauda	Ultrafiltration membranes	–	[87]
Spirulina sp.	Ultrafiltration membranes	20	[88]
Spirulina micractinium	Rotary vacuum filter	1–2	[89]
Spirulina	Belt filters	18	[8,90]
Haslea ostrearia, Skeletonema costatum	Crossflow microfiltration and ultrafiltration	–	[91]

TSS: Total suspended solids.

Key term

Costing: For the cost analysis, harvesting of 10,000 l of *Chlorella* sp. culture was considered and was calculated by using information from previously carried out studies and information provided by companies that supply the required chemicals and equipment to Brisbane (Australia).

was carried out to determine the technoeconomic feasibility of overall biomass recovery in a one-step as well as a two-step method. For the **costing** purpose, harvesting of 10,000 l *Chlorella* sp. culture was considered **(Table 3)**. Assessments were independently developed in accordance with Australian conditions and, where possible, were compared to equivalent costing from previous economic analyses of microalgal biofuel systems.

Table 3 compares some of the traditionally used harvesting methods in microalgal bioprocessing. From the table it can be summarized that due to its high energy consumption, single-step centrifugation is the most expensive method when compared with other techniques.

Flotation appears to be the most cost-effective method for primary dewatering; however, if used with centrifugation, the overall setup costs will increase and would result in higher capital costs. On the other hand, for flotation, if used in conjunction with filtration, the overall process may become more feasible but there is still room for improvement. Moreover, the cetyl trimethyl ammonium bromide chemical used for flotation is not only toxic to the environment but also makes the biomass unfit for human and animal consumption. Flocculation coupled with filtration may be more cost effective, but chitosan used for flocculation is biodegradable, as it is derived from a biological source (crustacean). However, large-scale use of chitosan may not be possible as it is expensive, as well as this putting pressure on crustacean populations. Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including *Pandalus borealis*. Hence, some harvesting techniques are more feasible than others when considering costs only, but some of these may not be environmentally friendly. Thus, there is a need to optimize current methods or to develop improved methods that are not only cost effective, but also environmentally friendly.

Classification of current harvesting processes

Current harvesting methods mentioned above can be divided into chemically based, mechanically based and biologically based **(Figure 2)**. Various combinations or sequences of these methods can be used for cost-effective harvesting. Currently, biologically based methods are being investigated as a cost-reducing and environmentally friendly means of harvesting [63]. In any case, it needs to be checked if any desirable valuable compounds are lost during the process. To develop a cost-effective harvesting technique, apart from the costs, one has to consider the following three main aspects: species-specific requirements of microalgae that need to be harvested; recovery/yield of desired product; and environmental impact.

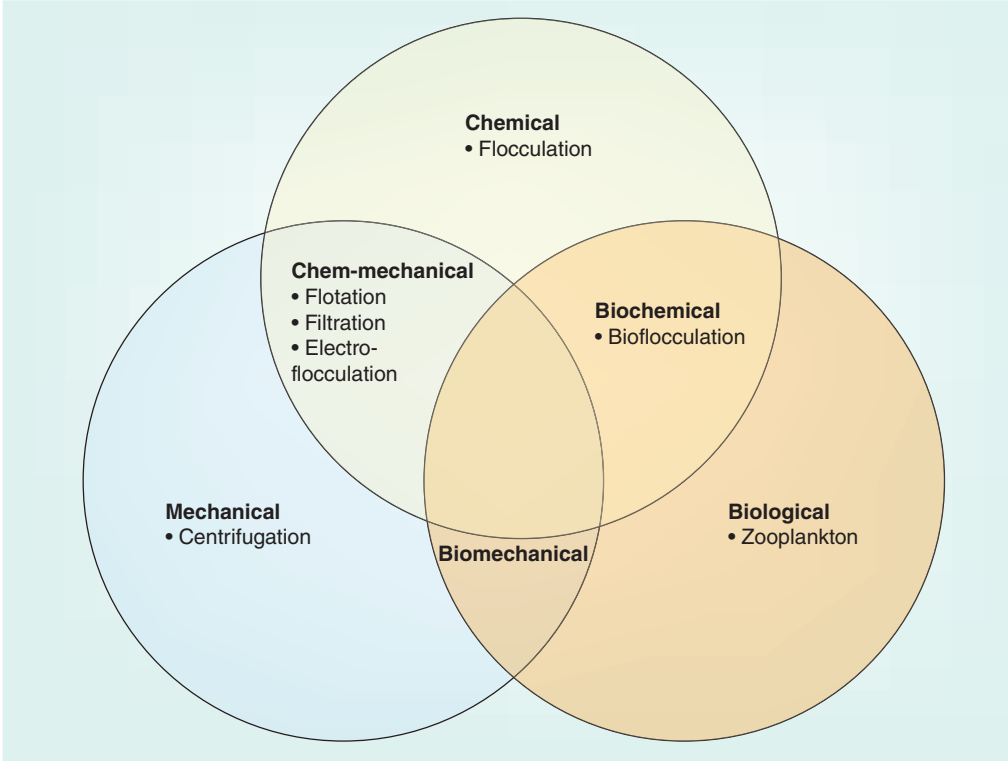

Figure 2. Interaction between different harvesting techniques.

Chemically based methods can be termed as a harvesting method that involves the addition of chemicals to the microalgae culture to induce flocculation, which is used in various solid–liquid separation processes as a pretreatment stage [64]. The chemical reactions are highly sensitive to pH, and high doses of flocculants are required to produce large amounts of sludge, which may leave a residue in the treated effluent. Although cost effective, a major disadvantage could be the presence of harmful salts and chemicals in the extracted biomass, which can possibly pose health and environmental risks. For example, use of aluminium oxide to flocculate microalgae can lead

to accumulation of aluminium salt precipitates in the biomass.

Mechanical harvesting, as the name suggests, is the method that involves the use of a mechanical machine to harvest microalgae, which generally includes centrifugation, filtration and flotation. Molina Grima *et al.* concluded that centrifugation is a preferred method for harvesting of microalgal biomass, especially for producing extended shelf-life concentrates for aquaculture, pharmaceuticals and other high-value products such as omega-3 [8]. However, Knuckey *et al.* state that exposure of microalgae cells to high gravitational and shear forces can damage the cell structure [28]. In addition, processing a large amount of culture using centrifugation is time consuming and increases the overall costs of microalgae biomass production **(Table 3)**. Filtration and gravitational sedimentation are widely applied in wastewater treatment facilities to harvest relatively large (>70 µm) microalgae such as *Coelastrum* and *Spirulina*. However, they cannot be used to harvest algae species approaching bacterial dimensions (<30 µm) such as *Scenedesmus*, *Dunaliella* and *Chlorella*, which can rapidly and easily blind the filter [16]. This may result in higher operating costs and frequent replacement of filters. In summary, most technologies including chemical and mechanical methods greatly increase operational costs for algal production and are only economically feasible for production of high-value products [65].

Biological harvesting is the method in which bioproducts or other microorganisms are used for the harvesting of microalgae. When cultivating microalgae, some cultures tend to aggregate and grow as fluffy pellets, or tightly packed, compact or dense granules. These fluffy pellets are caused by filamentous microorganisms, including some species of molds and bacteria [66–68], and may assist in trapping additional microalgal cells, one of the major advantages of cell pelletization [7,63,68]. Fungal cell growth can be induced by changing operational conditions during cell cultivation, rather than using $CaCO_3$ powder or other nuclei to induce the fungal pelletization [66], which are costly and cause solid waste disposal issues. A preliminary study was recently conducted by Zhou *et al.* to inoculate filamentous fungal spores when culturing mixotrophic green algae, *C. vulgaris*, with the result that pellets clearly formed within 2 days of culture [68]. Microalgal cells, aggregated together with fungal cells, were immobilized in the pellets [67]. Bioflocculation using flocculating microalgae has also been investigated by others [63,68]. The advantage of this method is that neither addition of chemical flocculants is required nor the cultivation conditions have to be changed. This method is as simple and effective as chemical flocculation; however, it is potentially more sustainable and cost effective. No additional costs are involved for pretreatment of the biomass before oil extraction and for the medium before it can be reused [27]. An interesting method is the use of zooplankton to harvest microalgae [69]. Biological harvesting could be a cost-effective method to harvest microalgae, but it is time consuming and has limitations in large-scale cultivation, as enough bioproduct must be co-produced. In addition, chances of cross-contamination are very high.

Based on our analyses conducted on microalgae harvesting technologies **(Table 3)**, it is evident that harvesting techniques should not only be cost effective and rapid, but also have to be environmentally safe and easily scalable for a microalgae-based biorefinery industry. Thus, there is a need to think outside the box and develop new hybrid methods that may combine the best aspects of several techniques **(Figure 2)**.

▪ Biochemically based methods

As described above, flocculation assisted by chitosan (biologically derived) has been used in many studies on different microalgae and has proven to be very promising [41,70,71]. Another example of biologically derived flocculation is the use of *M. oleifera* seeds, which have also been used for water treatment due to their high flocculation potential, low cost and low toxicity. Recently, Teixeira *et al.* demonstrated *M. oleifera* as a successful flocculating agent for *C. vulgaris* [44]. In addition, a range of new bioflocculants are proposed to address the cost and environmental concerns for current flocculation methods [14]. Microalgae flocculation was also achieved by using naturally available ions in brackish water, and a variety of precipitating ions, including Mg^{2+}, Ca^{2+} and CO_3^{2-}, can lead to autoflocculation of microalgae [32]. A combination of bioflocculants together with a low dose of chemicals may lead to the best flocculation outcome.

▪ Emerging technologies

When considering chemical, mechanical and biological harvesting methods, each method has its advantages and disadvantages. Biomechanical and chemical–mechanical methods for flocculation are less explored when compared with other methods. Developing hybrid techniques, which make use of all three harvesting categories, may be a viable option that is worth exploring.

The conceptual photobioreactor shown by Chen *et al.* has the potential to be developed into a commercially viable microalgae cultivation system with zero electricity consumption [56]. This was made possible by combining sunlight and multi-LED light sources with solar panels and a wind power generator. Similarly, when considering harvesting, electricity cost is the key factor that makes the process costly, but renewable energy sources such as solar and wind can be used to generate green

electricity [72]. The main disadvantage of these systems is the high construction costs.

Another option to reduce the cost of harvesting could be by combining two or more stages of microalgal biodiesel production with a harvesting method into one step; for example, as done in the study carried out by Taylor *et al.* [33]. By doing so, not only can the cost be reduced, but also the overall time required for a full production cycle. For example, developing a process that can help in rapid induction of lipids as well as flocculation could accelerate the harvesting process. Similarly, a method was developed by Hejazi *et al.* for milking β-carotene from *Dunaliella salina* in a two-phase bioreactor [73]. In this technique, cells were first grown under normal growth conditions and then stressed by excess light to produce larger amounts of β-carotene, and later a biocompatible organic phase was added and the β-carotene was extracted selectively via continuous recirculation of a biocompatible organic solvent through the aqueous phase containing the cells. Because the cells continue to produce β-carotene, the extracted product was continuously replaced by newly produced molecules. Therefore, the cells are continuously reused and do not need to be grown again. Thus, in contrast to existing commercial processes, this method does not require harvesting, concentrating and disruption of cells for extraction of the desired product [73,74].

Matrix-attached algae culture systems have been developed for growing microalgae on the surface of polystyrene foam to simplify the cell harvest [7,75]. These methods are innovative and will decrease the harvesting costs to some extent if developed successfully, but require heavy investments on equipment and chemical supplies with various combinations or sequences of these methods. Xu *et al.* developed a simple and rapid *in situ* magnetic harvesting method by using Fe_3O_4 nanoparticles on *B. braunii* and *Chlorella ellipsoidea*. Magnetic particles were added to the microalgal culture broth and then separated by an external magnetic field [76]. Recently a genetically modified approach has also been used for harvesting microalgae of genera *Chlamydomonas*, *Dunaliella*, *Scenedesmus* and *Hematococcus* sp. [76].

Conclusion

When considering the research carried out in the field of harvesting microalgae over the past few decades, much progress has been made. Researchers have optimized various techniques; machines have become more energy efficient. There is a need to optimize current methods or to develop improved techniques that are not only cost effective, but also environmentally friendly. Moreover, there is a need to develop hybrid harvesting technology that can use the best of all current harvesting methods. The costing calculation in this review suggests that flotation for primary dewatering coupled with filtration maybe the most cost-effective method for microalgal harvesting, but this may be different for different microalgal strains. The comparison also highlights the fact that none of the harvesting methods are cost effective when considering cultivation of microalgae solely for biodiesel production. Hence, it is a necessity to derive a secondary product that has a higher market value when compared with biodiesel. In the past, the majority of studies have focused on freshwater microalgae species and not much work has been done on marine species. With limited availability of freshwater, further research should be focused more on the processing of marine microalgae.

Future perspective

Rapid depletion of fossil fuels and rising GHG emissions have made the case of microalgae as a biofuel source even more compelling. Moreover, microalgae grown on nonarable land have great potential for provision of animal feed, and microalgae can also be used for wastewater purification. At present, harvesting technologies are costly and labor intensive, but recent studies indicate that major efforts are underway to develop new, more efficient and cheaper harvesting technologies, many of which will be microalgae strain specific. Microalgae are being grown in outdoor ponds, greenhouses, photobioreactors, fermenters and hybrid systems combining bioreactors and ponds. As more and larger microalgae pilot plants will be in operation within 5–10 years, more accurate economic assessments of different harvesting methods will be possible that will feed into the life cycle analyses of future algal biorefineries. With the availability of new and more efficient harvesting systems, microalgae harvesting will be less costly, easier to manage and more accessible for farmers, rural communities and industry around the world. Microalgal biorefineries are expected to be first established on a large scale in countries with high irradiation, flat, nonarable, desert, saline or low-biodiversity land, and access to water unsuitable for human consumption or irrigation (brackish, marine or polluted).

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Executive summary

Background

- Microalgae harvesting/dewatering is a major obstruction to industrial-scale processing of microalgae for biofuel production.
- When considering commercial-scale processes in order to dewater and prepare algal biomass for further downstream processing, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting and second is known as secondary dewatering/thickening.

Classification of harvesting techniques

- Primary harvesting includes flocculation, flotation and sedimentation. Secondary harvesting includes centrifugation and filtration.
- Current harvesting methods can be classified into chemically, mechanically and biologically based categories.

Costing & analyses of current harvesting techniques

- Single-step centrifugation is the most expensive method when compared with other techniques.
- Flocculation coupled with filtration may be cost effective, but chemicals used in flocculation of microalgae may lead to environmental damage.
- Flotation could be the most cost-effective method for primary dewatering; however, if used with centrifugation the overall setup costs increase and result in higher capital costs.

Future perspective

- There is a need to think outside the box and develop new hybrid methods that use best-of-all techniques and are not only cost effective, but also have a low environmental impact.

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UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production†

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Microalgae are highly efficient primary producers that can be grown in most types of water on non-arable land as a promising source of biofuel. However, large-scale microalgal biofuel production is currently uneconomical due to slow growth of high-percentage oleaginous algae and large harvesting costs. Here we present a new strategy, using a small dose of externally applied UV-C radiation, that significantly increases lipid contents of fast growing microalgae and that at higher doses also results in rapid settling. The procedure essentially separates biomass growth from lipid accumulation and harvesting which was tested in several microalgal strains and optimized to be completed within 48 h for *Tetraselmis* sp. using pilot-scale outdoor cultivation. This process resulted in a significant increase of both volumetric and areal lipid productivity with higher polyunsaturated fatty acid contents, while considerably reducing harvesting costs. Other benefits include control of co-cultured microbes and sanitized water for recirculation. UV-C-mediated lipid induction and settling (LIS) may contribute to commercial microalgal biofuel production.

Introduction

With growing tension regarding arable land and irrigation water resources, the argument for algae as a future food source, CO_2 sequestration medium, and biofuel supply becomes more compelling.[1–4] The new technologies need to produce an additional 5–6 billion tons of organic carbon apart from agricultural crops.[5] Large-scale microalgae cultivation is considered one of the most promising feedstocks to produce biofuels without competing for food production or biodiverse natural landscapes; recent progress and future perspectives have been intensively reviewed.[3–7] Microalgae can also be cultivated in brackish or seawater and as an integrated concept with wastewater treatment to optimize the energetic, nutrient and financial input for feedstock production.[8] However, high capital costs, low lipid productivity of fast-growing microalgae and high harvesting and processing costs are major bottlenecks hindering commercial production of microalgal oil-derived biodiesel. To obtain high lipid contents in fast-growing microalgae, typically lipid induction techniques need to be applied, but these may slow down growth and add extra costs.[9] Harvesting is another critical and challenging stage in mass microalgae cultivation due to low biomass concentrations (typically 0.3–0.5 g dry weight (DW) L^{-1}),[10,11] large volumes of water, and small cell sizes.[12–16] Harvesting consumes 20–30% of biomass production costs,[13,17,18] is alga-specific and may comprise different physical, chemical and biological processes.[11,12,18,19]

Synthesis and accumulation of large amounts of triacylglycerides (TAG) in microalgae is required for biodiesel production by transesterification. Cellular lipid and TAG induction techniques (either acting individually or in combination) include nutrient stress, osmotic stress, radiation, pH and temperature change, heavy metals and other chemicals, and some metabolic engineering approaches.[9,20–26] Nitrate starvation is most widely studied in almost all candidate biofuel microalgae[9] and is easily-applied by omitting nitrate from the growth medium or letting the culture use up nutrients. However, it typically takes 3–5 days until significant amounts of lipids are synthesized, which is accompanied by slow growth rates and thus finally affects the total biomass and lipid productivity.[27] Changes in temperature, pH, salinity and heavy metals are difficult to regulate on a large scale. Genetically-modified microalgae potentially produce more lipids, but regulatory issues increase costs, while microalgae naturally high in lipids or hydrocarbons are typically slow-growing.[28]

Thus there is a demand for a process that can not only rapidly induce lipids in exponentially growing algae, but also bridge the gap between the time taken in microalgal biomass

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production and harvesting the biomass. After considering different lipid induction techniques, we concluded that external lipid induction with light irradiation is a promising approach that does not involve any alternation in growth media, or leave traces in the biomass and that, moreover, can be easily dosed. Solar ultraviolet light comprises UV-A (400–315 nm; 3.10–3.94 eV per photon), UV-B (315–280 nm; 3.94–4.43 eV per photon), and UV-C (280–100 nm; 4.43–12.4 eV per photon), but the latter does not reach the Earth's surface. UV-A and UV-B have already been tested for microalgae, but lipid induction took more than 2–3 days to show an effect.[22,24,29–31] As UV-C light carries more energy per photon, the hypothesis was to use UV-C (253 nm) radiation as a stress induction technique after reaching a certain cell density, in order to minimize biomass loss and obtain high lipid productivity. Several microalgae were tested and the oleaginous marine microalga *Tetrasemis* sp. M8 has been chosen as the main strain for this study, based on its previous identification as a candidate strain for biofuel feedstock, including comparatively rapid dominant growth under mid-scale outdoor conditions (growth rate μ = 0.47) and a polyunsaturated fatty acid (PUFA) profile suitable for biodiesel production.[32] Our results demonstrate that UV-C stress not only led to doubling of cellular lipid contents but also led to the loss of flagella and subsequent settling, a convenient, time-saving and cost-effective method for microalgae harvesting.

Methods

Laboratory-scale microalgae culturing and UV-C treatment

Strain *Tetraselmis* sp. M8 was collected in an intertidal rock pool at Maroochydore, Australia (26°39′39″S, 153°6′18″E; 12 pm on 6 August 2009[32]). Pure cultures of the isolate M8 were grown in f/2 medium[48] in autoclaved artificial sea water (35 ppt NaCl). *Tetraselmis chui*, *Chlorella* sp. BR, *Nannochloropsis* sp. BR2, and *Dunaniella salina* strains have been previously described.[32] Laboratory culturing conditions were set at 23 °C on an orbital shaker (Thermoline) at 100 rpm with 12 hours of light and dark rhythm. When the cell density reached 10^6 mL^{-1}, the culture was used for UV-C radiation trials. Primary stock cultures were maintained aerobically in 100 mL Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25 °C, under a 12 : 12 h light–dark photoperiod of fluorescent white light (120 μmol photons m^{-2} s^{-1}). Nitrate and phosphate levels were determined using the corresponding API Nutrient testing kit according to the manufacturer's instructions. After gently stirring, 5 mL aliquots of M8 culture were pipetted into a Petri dish, forming a thin layer inside (a total of 20 plates). Plates were randomly divided into five groups with three plates used for each UV-C radiation (253 nm) treatment in a UV chamber (BioRAD, Gs-Genelinker). They were separately irradiated at 0, 100, 250, 500 and 1000 mJ cm^{-2}. All Petri dish cultures were subsequently incubated for 24 h. Then the algae survival rate was measured by counting the live cells based on visibly intact chloroplasts in each replicate. The cell size was also measured by compound microscopy (Olympus).

Lipid fluorescence analysis

For each replicate, 1 mL of algae cells was sampled and stained with 3 μL Nile Red solution (a 10 mg mL^{-1} Nile Red (Sigma, USA) stock solution was prepared in acetone and stored in the dark at 4 °C). After 20 min incubation in the dark, the lipid fluorescence intensity of cells was detected by fluorescence-activated cell analysis (BD LSR II: analyzing flow cytometer) with 573 nm excitation wavelength. A total of 10 000 cells were counted in each sample. A gate was set up to separate the fluorescence-activated cells and inactivated cells based on the analysis of cells without Nile Red staining. The background absorbance from chloroplast auto-fluorescence was subtracted from each sample using mock-treated (control) *Tetraselmis* sp. M8. Qualitative observation was conducted simultaneously by fluorescence microscopy as described below. For plate reader analyses, samples were stained with Nile Red stock solution as mentioned above and 200 μL of each sample was loaded into 96 well plates and analyzed with a Fluostar optima and Polarstar optima (BMG LAB tech) plate reader at excitation and emission wavelengths of 485/584 nm; the gain was set at 2400.

GC-MS analyses

GC-MS analysis was carried out as described previously.[32] Briefly, 4 mL of algal culture was centrifuged at 16 000*g* for 3 min. The supernatant was discarded and the lipid in the algal pellet was hydrolyzed and methyl-esterified with 300 μL of 2% H_2SO_4 methanol solution at 80 °C by shaking (480 rpm) for 2 h on a thermal mixture (Eppendorf). Prior to esterification, 50 μg of heneicosanoic acid (C21) was added to the pellet in each sample as an internal standard (IS). After esterification, 300 μL of 0.9% (w/v) NaCl and 300 μL of hexane (analytical grade) were added and vortexed for 20 s. Phase separation was performed by centrifugation at 16 000*g* for 3 min and the hexane layer was used for lipid profile analysis. GC/MS analyses were carried out on an Agilent 6890 GC coupled to a 5975 MSD. A DB-Wax column (Agilent, 122–7032) was used with running conditions as described in Agilent's RTL DBWax method (application note: 5988-5871EN). Identification of fatty acid methyl esters was based on mass spectral profiles and retention times in Agilent's RTL DBWax method. Each fatty acid methyl ester (FAME) was quantified using the formula:

$$\text{Fatty acid } (\mu g\ mL^{-1}) = (\text{integral of fatty acid/integral internal standard}) \times (\text{molecular mass of fatty acid/molecular mass of internal standard}) \times 50/4$$

Enzymatic assays

Lipid peroxidation, superoxide dismutase (SOD) and glutathione reductase (GR) activities were determined using the

TBARS, Superoxide Dismutase or Glutathione Reductase (GR) Assay Kits, respectively, (Cayman) according to the manufacturer's instructions. A total of 5 mL culture with 10^6 cells mL^{-1} was used as UV-C treated cells (0–1000 mJ cm^{-2}) and a mock-treated control culture were centrifuged at 2000*g* and resuspended in 1 mL diluted assay buffer provided by the manufacturer. The cultures were then sonicated three times for 5 min at 40 V setting on ice, centrifuged at 1500*g* for five min at 4 °C and the supernatant was used to perform the assay.

UV-C-mediated microalgal lipid biosynthesis induction and settling (LIS)

A total of 20 L of exponentially-grown culture of *Tetraselmis* sp. M8 were taken from a 1000 L raceway pond and poured in two 50 L aquarium fish tanks, each containing 10 L of culture (ESI Fig. 5†). One of the tanks was UV-C-radiated for 15 min (3 J cm^{-2}) with a custom-built UV-C chamber with continuous bubbling to ensure that all cells were evenly radiated, while the other tank was a mock-treated control culture without UV-C radiation that was bubbled for the same time. A total of 5 mL of the culture from both the tanks was taken into 20 mL Falcon tubes for Nile red-staining and cell counting. The culture was left overnight for settling and lipid induction. Cell counting was carried out at 3 h and 15 h after treatment.

Outdoor raceway cultivation

In order to evaluate the UV-C lipid induction and settling technique of microalgae in a mid-scale outdoor setting, 20 L of laboratory-grown *Tetraselmis* sp. M8 culture was used to inoculate two identical 1000 L outdoor raceway ponds built by the University of Queensland's Algae Biotechnology Laboratory (http://www.algaebiotech.org; ESI Fig. 7†). Mid-scale outdoor raceway experiments were conducted between January and September 2012, with most experiments carried out during winter time under sunny conditions when average day temperatures ranged from 22 °C to 26.5 °C. Cultures were continuously grown in 1000 L outdoor raceway ponds and climatically adapted before experiments commenced. Cultures were continuously grown in seawater containing f/2 medium at uncontrolled pH values of 8.8–9.1 and cell culture densities of 1.5–2.3 × 10^6 mL^{-1}. Cell counts were conducted daily followed by Nile red fluorescence and GC-MS analyses during controlled experiments. Cultures were also checked daily under the microscope to ensure that no contamination with other microalgae occurred.

After initial optimization experiments in the outdoor raceway ponds, 500 L of microalgae culture with 12 cm water depth was used for 6 h (72 J cm^{-2}) of UV-C treatment. This dosage was found suitable to induce lipid biosynthesis with minimum cell death. Subsequent experiments were set up for 8 days out of which typically by days 3 and 4 the culture was under N starvation and after which an increase in fluorescence of Nile red-stained cells could be measured. To further minimize the initial cell mortality, the UV-C dosage was divided into two phases. An initial dose of UV-C was applied on day 5 for 4 h followed by 36 h continued cultivation for rapid lipid induction. A final dose of UV-C was applied for 2 h at the end of day 7, culture-mixing was stopped and the culture was harvested the next morning after overnight settling. The experiment was repeated three times and included a raceway swap, but included a reduced frequency of measurements.

Dry weight measurement

At the end of outdoor cultivation, just before harvesting, 25 mL of the culture was used for dry weight measurements. The 25 mL culture was filtered through a 0.27 μm glass fiber filter (Millipore) which was pre-weighed and pre-washed with 1 mL distilled water in a vacuumed filter unit (three biological replicates were used from each culture (UV-C treated and mock-treated control)). After filtration the filters were kept in individual Petri dishes to avoid contamination and dried in a drying oven for 24 h at 80 °C with the plate lid half open. After 24 h the filters were immediately weighed.

To determine the dry weight the following formula was used:

$$\text{Dry weight mg L}^{-1} = \frac{\text{Filter dry weight} - \text{Filter pre-weight}}{\text{Filtered volume mL} \times 1000}$$

Dry weight in mg L^{-1} was determined from the average of three weight measurements for each replicate.

Microscopic analyses

After a lipid induction phase, microalgae cells were stained with 2 μg mL^{-1} Nile red (dissolved in acetone; Sigma, USA) for 15 min and photographed using a fluorescent Olympus BX61 microscope fitted with a 100 W High Pressure Mercury Burner and an Olympus DP10 digital camera. Differential interference contrast (DIC) and epifluorescent (excitation: 510–550 nm, emission: 590 nm) images were obtained at 1000× magnification with oil immersion.

UV-C disinfectant effect

In the morning on day eight of the experiment, just before harvesting, the top layer of both the raceways was collected and 100 μL of the culture was spread on LB plates and PDB plates to screen for bacteria and fungal colonies present after the experiment. The bacterial plates were incubated in a 37 °C growth chamber for 48 h, whereas fungal plates were incubated for 7 days at room temperature. Microbial growth was monitored every 24 h.

Analytical methods

Measurement of nitrate and phosphate levels in the photobioreactor was performed using colorimetric assays (API, Aquarium Pharmaceuticals and Nutrafin, respectively). Data for growth rates and lipid productivity were statistically analyzed by one-way analysis of variance (ANOVA) with different microalgal cultures as the source of variance and the growth rate or lipid productivity as dependent variables. This was followed by Tukey's multiple comparisons test ($P > 0.05$) where appropriate. Student's *t*-test was used for pairwise comparisons.

Table 1 Costs of harvesting 10 000 L of *Tetraselmis* sp. culture with different harvesting techniques

	Single step dewatering Centrifugation	Primary dewatering			Secondary dewatering	
		Sedimentation	Flotation	LIS UV-C	Filtration	Centrifugation
Total energy consumed	55 kWh per 10 m^3[c]	—	7.4–8.4 kWh per 10 m^3[d]	5.76 kWh per 10 m^3	1–3 kWh m^{-3}	5.5 kWh m^{-3}[c]
Time required (hours)	10	—	2	6	1	1
Energy cost (AUD)[a]	$12.10[b]	—	$1.62–$1.84[b]	$1.26[b]	$0.22–$0.66[b]	$1.21[b]
Dosage required	—	400 g @ 40 mg L^{-1} [46]	800 g @ 80 mg L^{-1} [47]		—	—
Chemicals (AUD)	—	$10 (chitosan @ $25 kg^{-1})	$6.4 (CTAB @ $8 kg^{-1})	—	—	—
pH Adjustment dosage	—	1.5 to 2 L acetic acid[e]	—	—	—	—
pH Adjustment cost	—	$1.20–$1.60 @ $800 t^{-1}	—	—	—	—
Total costs (AUD)	**$12.10**	**$11.10–11.20**	**$8.02–$8.24**	**$1.26**	**$0.22–$0.66**	**$1.21**

[a] Australian dollar (= approx. US$1.04) [b] Electricity prices were calculated based on $0.22 kWh^{-1}. [c] An Evodos (E-25) centrifuge was used for this study (at the flow rate of 1000 L h^{-1}). [d] The flotation cell considered is a Jameson cell and energy consumption was determined using various published studies.[47] [e] The volume was estimated by conducting an experiment with 1 L of algae culture and mathematical calculation. Values may differ for different algal strains as these may have different preferential harvesting conditions.

Construction of a UV-C chamber

For scaling up the UV-C lipid induction and settling technique, a UV-C chamber was custom-built using a stainless steel hood of a plant growth chamber light lamp in which the original fluorescent tubes were replaced with six G8T5 (TUV 8W) GE Philips Bi-Pin 288, UV-C (253 nm) lamps connected in series (ESI Fig. 5A†). The height, length and width of the chamber were 40 cm, 45 cm and 40 cm, respectively. Based on a comparison with the specifications of the laboratory UV chamber (BioRAD, Gs-Genelinker), the heat losses of the lamps and the set-up of the outdoor chamber, 12.5% of the energy was estimated to reach the culture surface as UV-C radiation (approx. 12 J cm^{-2} h^{-1}; the actual UV-C surface radiation was not measured).

Techno-economic analysis

Using the time taken to harvest 1000 L of *Tetraselmis* sp. M8 culture in mid-scale outdoor cultivation and information from previously carried out studies and specifications provided by companies that supply the equipment and chemicals, a theoretical calculation was carried out to determine the techno-economic feasibility of overall biomass recovery in a one-step as well as a two-step method. For the costing purpose, harvesting of 10 000 L of *Tetraselmis* culture was considered (Table 1). Assessments were independently developed in accordance with Australian conditions and, where possible, were compared to equivalent costing from previous economic analyses of microalgae biofuel systems.[11]

Results

UV-C irradiation induces microalgal lipid biosynthesis

Microalgae have been suggested to be a promising source of triacylglycerides for biodiesel production. *Tetraselmis* sp. M8 microalgal cells had previously been shown to accumulate significant amounts of lipids after nutrient deprivation.[32] Preliminary experiments using UV-C radiation on various microalgal cells, including *Tetraselmis* sp. M8, indicated that this external stress can also stimulate lipid accumulation (ESI Fig. 11†). To test whether both, nutrient starvation and UV-C treatment, can lead to further lipid biosynthesis, combined sequential stress treatments were carried out. Nile red-staining of nitrogen-deprived *Tetraselmis* sp. M8 cells indicated that an additional external stress treatment by UV-C exposure led to an increase in cell sizes and additional lipid accumulation in lipid bodies within 24 h (Fig. 1). As UV-C doses were increased from 100 mJ cm^{-2} to 250 mJ cm^{-2}, fluorescence intensities became stronger, but at higher doses cell rupturing was observed. Interestingly, detached flagella were found at UV-C radiation doses at and above 100 mJ cm^{-2} (arrow in Fig. 1a) and this could also occasionally be observed under a fluorescence microscope with blue light excitation within a few seconds. As expected, a sharp decline in cell survival rates was observed with increased UV-C radiation (Fig. 1b). The cell survival rate was approximately half (LD_{50}) at 250 mJ cm^{-2} (Fig. 2).

To profile and quantify lipid accumulation of millions of individual nutrient-starved *Tetraselmis* cells simultaneously following different UV-C exposure, a flow cytometer was used (Fig. 2). With an increase of UV-C radiation, algal cells were divided into two different populations, termed P1 and P2, according to a background fluorescence intensity cutoff in the unstained control population. The majority of the P1 population in this control can be attributed to auto-fluorescence of chloroplasts (ESI Fig. 1a†). In the untreated, but Nile red-stained control, 60% of the cells were allocated to the P1 population, whereas 40% were in the P2 population (Fig. 2a; ESI Fig. 1b†); the latter can be attributed to lipid accumulation by nutrient deprivation in these cells. When UV-C radiation of 100 or 250 mJ cm^{-2} was used for treatment of nutrient-starved cells, P1 was reduced to 40%, while the lipid-accumulating P2 increased to 60%, indicating a marked increase in lipid pro-

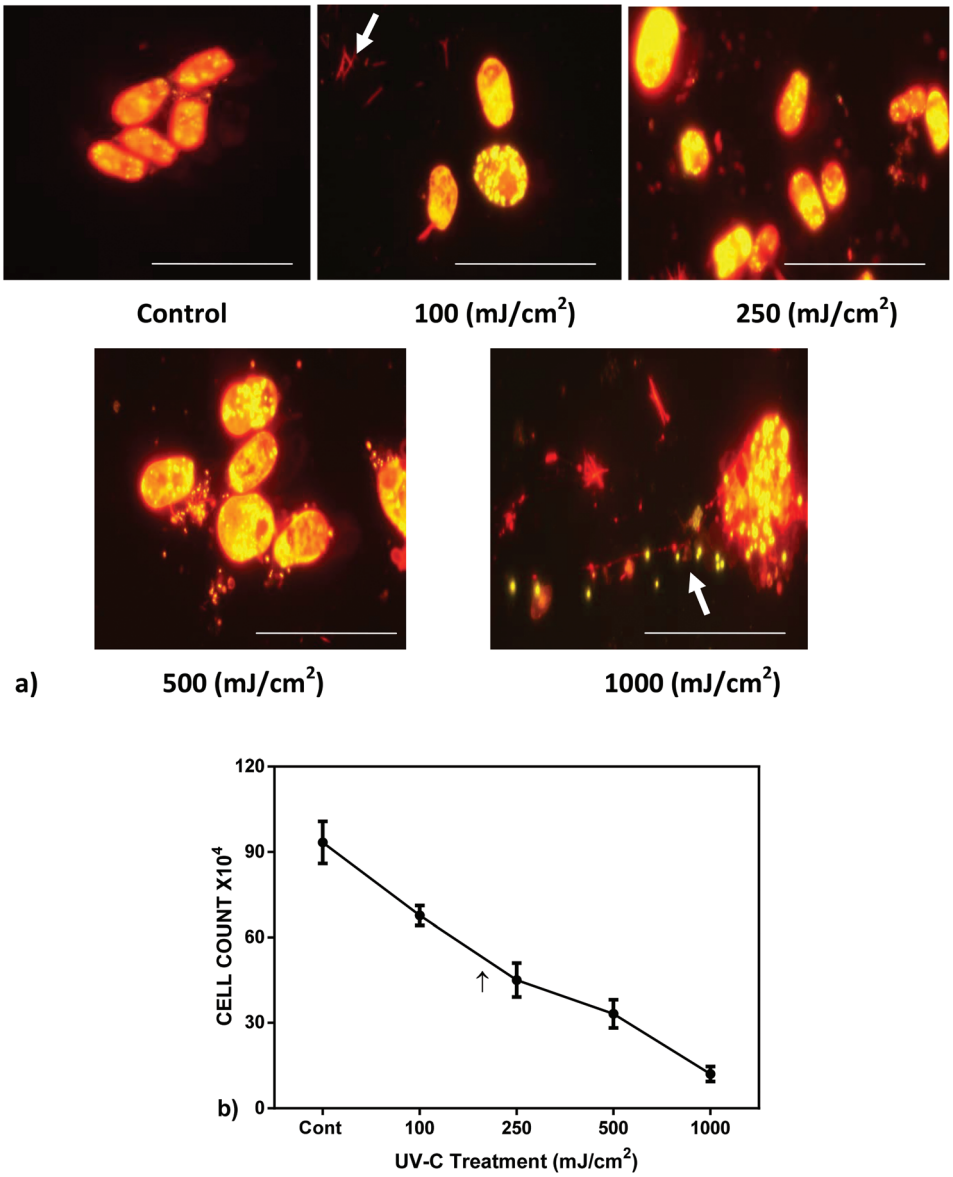

Fig. 1 Nile red-stained cells of *Tetraselmis* sp. M8 that received different doses of UV-C exposure. (a) Cells with maximum lipid fluorescence (yellow) can be observed at 250 mJ and 500 mJ UV-C radiation. Starting from UV-C of 100 mJ cm^{-2} detached flagella can be observed (white arrow); as the radiation is increased to 1000 mJ cm^{-2} cell rupturing occurs (white arrow) with lipid bodies released in the medium. Cells are shown at 40× magnification (bar = 50 μm) at 24 h after treatment. (b) Kill curve of *Tetraselmis* sp. M8, showing the number of cells that survived UV-C treatment at different dosages. Shown are mean values ± SEs from three independent treatments of M8 culture. The arrow indicates the LD_{50} value.

duction after UV-C treatment (Fig. 2a; ESI Fig. 1c and d†). However, at 500 mJ cm^{-2} P1 and P2 were equal and at 1000 mJ cm^{-2} P1 further increased to 65% while the high lipid-containing P2 decreased to 35% (Fig. 2A; ESI Fig. 1e and f†). This is consistent with microscopic observations suggesting that high UV-C doses damaged cells to a point where most cells were unable to produce Nile red fluorescence-detectable lipids. Quantification based on average fluorescence intensities from three separately-grown and -treated microalgal populations showed that P1 cells always had much less fluorescence intensities than the lipid-accumulating P2 populations (Fig. 2b). Overall, fluorescence intensities of both populations combined confirmed that nutrient-starved UV-C-treated cells (100 mJ cm^{-2}) had significantly increased lipid contents (P = 0.022, Fig. 2c). However, there was no significant fluorescence increase between the untreated control and cells treated with 250 mJ cm^{-2} or higher doses which can probably be attributed to cell damage leading to cell rupturing and release of lipid bodies that were not quantified by flow cytometry.

Fatty acid profiles of UV-C-treated microalgae shift towards unsaturated fatty acids

To further quantify the ability of UV-C stress to increase cellular TAG contents and to profile the fatty acid composition,

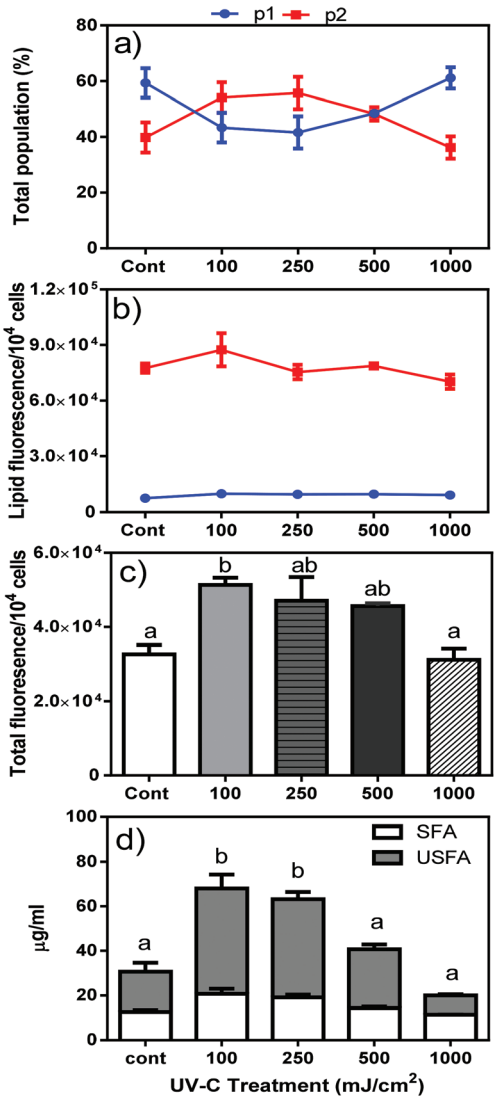

Fig. 2 Lipid induction in *Tetraselmis* sp. M8 cultures at 24 h after receiving different UV-C dosages. (a–c) FACS analysis of Nile red stained cells showing distribution (a) and lipid fluorescence (b) of low (P1) and high (P2) fluorescence cell populations and of the total population (c). (d) Triacylglyceride quantification by gas chromatography-mass spectroscopy (GC-MS) showing total fatty acids as well as saturated (SFA) and unsaturated (USFA) fatty acids produced by different UV-C-treated cultures. Shown are mean values ± SEs from three separately-grown and treated microalgae cultures. Bars with different letters indicate significant differences ($P < 0.05$). See ESI Fig. 1† for the corresponding scatter plots of P1 and P2 populations and ESI Fig. 2 and 3† for graphs of individual fatty acids of microalgae treated with different UV-C dosages.

GC-MS analyses were performed. These confirmed the results obtained from flow cytometry showing a significant total fatty acid increase ($P = 0.032$, $P = 0.014$, respectively) of UV-C-treated cultures (100 and 250 mJ cm^{-2}) compared to untreated controls, whereas cultures treated with 500 and 1000 mJ cm^{-2} showed no significant difference (Fig. 2d). Moreover, in cultures treated with 100 and 250 mJ cm^{-2}, the amount of unsaturated fatty acids (USFA) significantly increased and also the proportion of USFA compared to total fatty acids (Fig. 2d). Amongst the saturated fatty acids (SFA), the highest increase was observed for C16 (palmitic acid), followed by C14 (ESI Fig. 2†). On the other hand, C18 and C20 did not show any increase in cultures treated with 100 and 250 mJ cm^{-2} UV-C, whereas in cultures treated with 500 and 1000 mJ cm^{-2} the amount of C20 was significantly higher ($P = 0.0239$, $P = 0.0164$, respectively). When comparing different USFA, cultures treated with 100 and 250 mJ cm^{-2} UV-C showed significant increases for all detected USFA, most notably C16:4, C18:1 *cis* + *trans*, C18:3n3 and C20:5 (ESI Fig. 3†).

It appears plausible that UV-C stress leads to cellular lipid biosynthesis to improve survival of microalgal cells, as has been observed for many other stresses, most of which led to a higher proportion of SFAs.[9] To better understand the underlying mechanisms for the observed shift of fatty acids towards unsaturated fatty acids following UV-C treatment, several enzymatic assays were carried out. Decomposition of unstable peroxides derived from PUFAs resulting in the formation of malondialdehyde (MDA) was quantified colorimetrically following its controlled reaction with thiobarbituric acids (TBARS). ESI Fig. 4a† shows that as the UV-C radiation increased from 0 to 1000 mJ cm^{-2} the formation of MDA increased in samples and was highest at 1000 mJ cm^{-2}. Similarly, superoxide dismutase (SOD) and glutathione reductase (GR) activities increased with higher UV-C doses (ESI Fig. 4b and c†), suggesting that UV-C-treated cells underwent oxidative stress causing damage to DNA and cellular membranes. Hence an increased amount and proportion of USFA in UV-C-stressed cells may be required for repair of cellular damage to membranes and to provide sufficient antioxidant capacity to restore redox homeostasis.

UV-C treatment leads to settling of flagellate algal cultures

While conducting UV-C lipid induction experiments with cultures of the flagellate microalgae *Tetraselmis* sp. M8, it was observed that cells exposed to UV-C radiation also detached their flagella and showed other signs of cell damage (Fig. 1). The effect could also be observed under blue light using fluorescence microscopy and for other flagellate microalgae (data not shown). When applied to entire laboratory-grown cultures, this resulted in settling of the algal cells if left undisturbed. Hence the notion of combining lipid induction with settling for microalgae harvesting was developed. The process of exposing flagellate algal cultures to UV-C irradiation is therefore hereafter referred to as Lipid Induction and Settling (LIS). To test whether this is applicable to outdoor conditions, algal culture settling experiments were performed on saturated 20 L cultures grown in open-lid aquariums using a specially-designed UV-C chamber (ESI Fig. 5†). Within 3 h of LIS treatment, about 90% of the cells settled when compared to untreated cultures and within 15 h, >99% of the cells settled (ESI Fig. 6†).

Upscaling of lipid induction and settling (LIS)

Following initial optimization of UV-C-mediated LIS to determine a suitable dose of UV-C, experiments were conducted on

two pilot-scale 1000 L raceways ponds (ESI Fig. 7†). Both ponds were built with exactly the same specifications for optimal comparisons. Cultures were first pre-adapted to growth in these ponds for 2 months where they were maintained in late exponential growth phase. For the experiment, the same culture was split into two identically-built raceways and grown simultaneously where one of the raceways was UV-C-treated and the other was mock-treated. The trials commenced at a cell density of 1.5×10^6 mL^{-1} (day 1; Fig. 3). By day 3 cells had reached the nutrient depletion point (nitrate and phosphate not measurable) and days 4 and 5 were intended for nutrient stress induction. In the early morning on day 5, an initial dose of UV-C was applied for 4 h (48 J cm^{-2}). As a result, the cell count dropped slightly from 1.7×10^6 to 1.5×10^6 in the UV-C-treated raceway (Fig. 3a). After 36 h of lipid induction phase, a final dose of UV-C was applied in the evening of day 7 for 2 h (24 J cm^{-2}) to induce loss of flagella and settling and cells were left for overnight sedimentation. In the morning, more than 93% of UV-C-treated cells had settled and the remaining cell count of the culture dropped to 10^5 mL^{-1}, whereas the mock-treated raceway contained 1.5×10^6 cells mL^{-1} (Fig. 3b). Along with cell density, lipid fluorescence from Nile red-stained cells and fatty acid profiles were determined at different stages of the experiment (Fig. 4). From day 5, a clear increase in lipid fluorescence was observed, reaching a maximum on day 6 that was twice as high in the UV-C treated raceway (Fig. 4a). As the cell density in UV-C-treated cultures was slightly lower than that in the control, values for total lipids per cell were significantly higher in UV-C-treated culture on day 6 compared to the control (Fig. 4b).

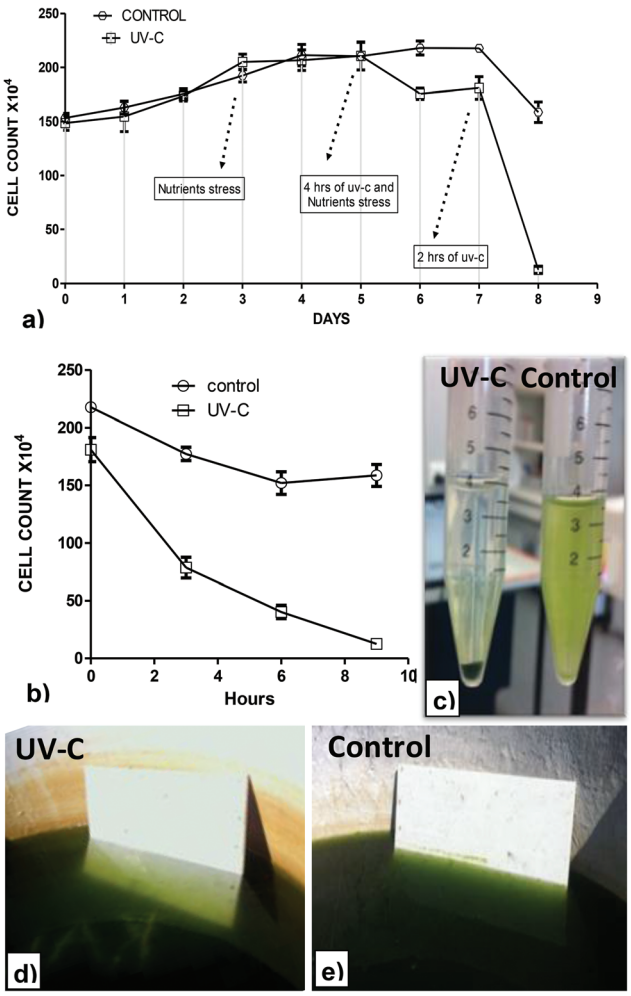

Fig. 3 Settling of control and UV-C-treated algal cultures in 1000 L airlift-mixed raceway ponds. (a) Cell counts indicating different phases of the experiment on days 3, 5 and 7. (b) Settling curve of control and UV-C-treated culture on day 8. (c) Settling of UV-C-treated and control culture. (d, e) Settling of UV-C-treated and control cultures, respectively, in 1000 L raceway ponds against the background of a white plate. Shown for a–c are mean values ± SEs from three measurements. The experiment was repeated three times with similar results.

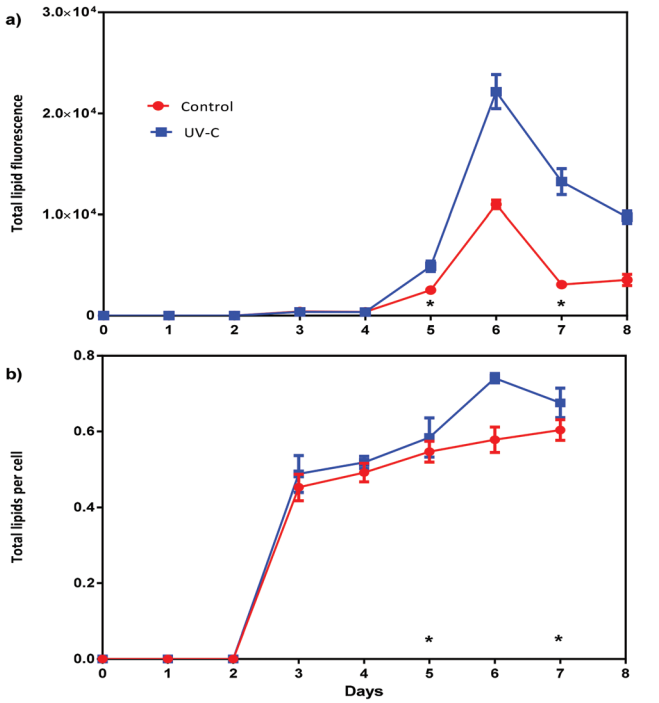

Fig. 4 Lipid fluorescence of Nile red-stained cells in control and UV-C-treated raceway ponds. Maximum lipid fluorescence was observed on day 6 which was an indication of UV-C lipid induction. Shown are total culture lipid fluorescence (a) and lipid fluorescence per cell (b) as mean values ± SEs (n = 3). Asterisks indicate the time points when UV-C treatment was applied.

As expected, the total DW of harvested biomass obtained from UV-C-treated raceways was marginally reduced compared to the control (Fig. 5a). However, the total fatty acid yield determined by GC-MS was significantly higher (300 μg mL^{-1}) when compared to the control (200 μg mL^{-1}; Fig. 5b), whereas the total lipid productivity was also significantly ($P < 0.05$) higher (35 mg L^{-1} per day) when compared to the control (23 mg L^{-1} per day) (ESI Fig. 8†). Consistent with the lab-scale studies, the highest increase in fatty acids could be attributed to USFA contents ($P = 0.028$), whereas the total SFA increase was not significant ($P = 0.058$; Fig. 5c and d). Specifically, consistent with the

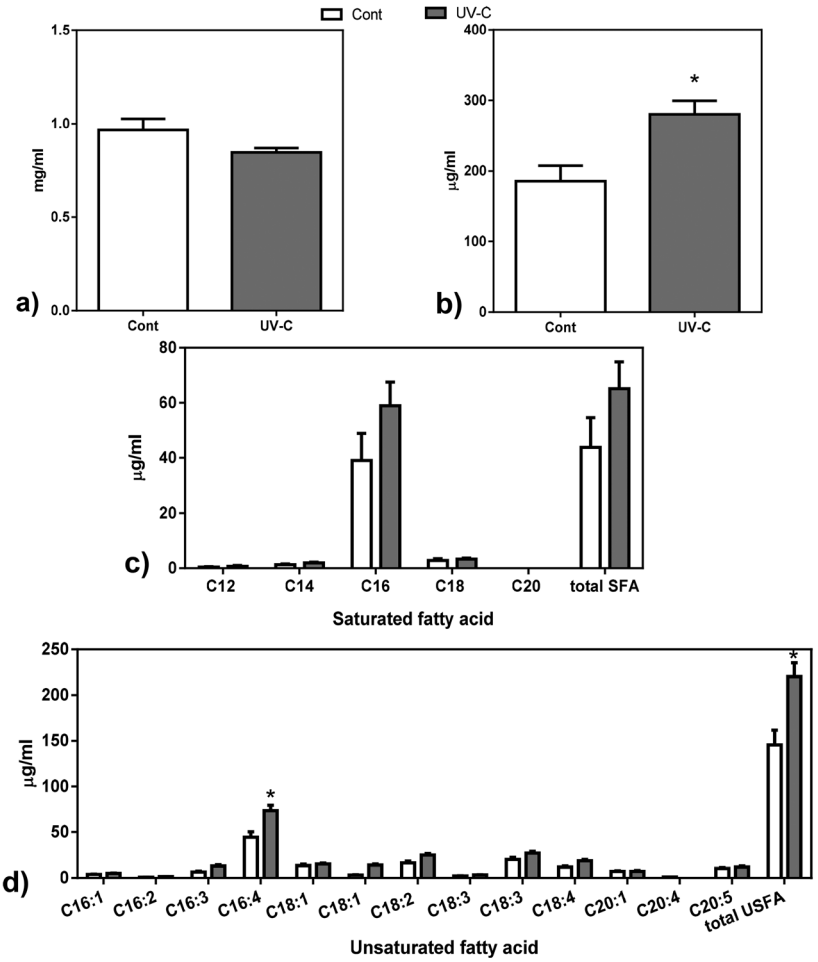

Fig. 5 Biomass dry weights and fatty acid profiles in harvested culture of control and UV-C-treated raceway-grown algal cultures. Shown are total dry weights (a), total fatty acid contents (b), as well as individual and total saturated (c) and unsaturated (d) fatty acid profiles. Bars with (*) show significant differences ($P < 0.05$). Shown are mean values ± SEs from three separately-grown and separately-treated raceway cultures.

lab-scale studies, the highest increases were observed for C16, C16:3, C16:4, and C18:4 (ESI Fig. 2 and 3†).

UV-C treatment aids in water sanitization for reculturing

To investigate whether UV-C treatment had an effect on bacterial and fungal growth in cultures, both groups of organisms were quantified in UV-C-treated *vs*. untreated used growth medium from outdoor raceway ponds. As expected, the UV-C-treated raceway pond harbored significantly-reduced culturable bacteria and fungi (ESI Fig. 9†).

Techno-economic analysis shows reduced costs for primary dewatering

A techno-economic analysis was carried out to determine whether the implementation of LIS would result in significant cost savings compared to other currently-used methods for algal biomass harvesting. As shown in Table 1, an estimated 89% and 45% of the costs for primary dewatering of *Tetraselmis* sp. M8 can be saved for flocculant-assisted settling or dispersed-air-flotation, respectively, when using LIS instead. When considering lipid productivity rather than algal biomass, LIS stood further out as a cost-effective method as it would further reduce costs on a per lipid basis. Alternatively, cost savings may be achieved by shortened lipid induction periods of nutrient- and UV-C-stressed cultures, compared to cultures stressed by nutrient depletion alone. Another advantage of LIS compared to flocculant-assisted methods is that no chemical residues remain in the harvested biomass. Full life cycle analyses should be conducted on optimized large-scale cultivation systems to determine how actual costs for algal biomass and lipid production compare to other feedstocks.

Discussion

This study first introduced UV-C radiation to stimulate lipid biosynthesis in microalgae. It highlights the efficiency of UV-C radiation at lipid induction and also provides a benchmark for UV-C-induced settling of flagellate microalgae. Maximum lipid induction was depicted by cultures radiated at 100 mJ cm^{-2} and 250 mJ cm^{-2} on Petri dishes (Fig. 2c and d), and 48 J cm^{-2} was found suitable for 12 cm deep *Tetraselmis* outdoor raceway

cultures containing 1.5×10^6 cells mL^{-1} (Fig. 4 and 5). UV-C-induced settling occurred overnight. It should be considered that UV-C may also cause an increase of mutations in the culture. To avoid adaptation to repeated UV-C exposure all the culture was harvested after the experiment.

Coincident with studies conducted with UV-B radiation,[33,34] an increase of cell size was found (Fig. 1a; ESI Fig. 1e and f†). A study on nitrogen deprivation in *Dunaliella tertiolecta* also noted increased cell size following lipid accumulation.[35] So it was understood that the cell size increased by UV-C as a result of lipid induction. However, larger cells may display higher UV stress tolerance as suggested by UV-B research.[33,34] Cellular lipids may simply provide more energy reserves for stress responses. Alternatively, a UV-induced morphological variation may be better adapted to adverse conditions/lipid production. Since UV causes genetic mutations,[36,37] UV may have acted as a selection pressure during algal evolution.[38,39] At high UV-C doses, the release of lipid bodies became apparent as cell structures disintegrated (Fig. 1). Considering the difficulties often experienced for lipid extraction due to rigid cellular structures, UV-C-implemented cell damage may also contribute to higher lipid extraction efficiencies.

Compared to previous lipid induction techniques,[9,40,41] UV-C radiation resulted in faster lipid biosynthesis stimulation within 24 h (Fig. 2–4). Depending on the quantification method used, the total lipid or TAG production approximately doubled compared to lipid induction by nutrient depletion only. Under outdoor conditions, TAGs were induced to a lesser extent (Fig. 5b), perhaps because controls also received UV-containing solar irradiation or because biomass was harvested after the peak in lipid fluorescence of Nile red-stained cells (Fig. 4a). This emphasizes that further fine-tuning of UV-C exposure and harvesting times may be required to maximize TAG induction while minimizing cell mortality, and this is likely to vary for different microalgae, cultivation systems, and climatic conditions.

Although the total lipid content increased by UV-C radiation to different extents, an alteration of fatty acid profiles only occurred at low doses (100 and 250 mJ cm^{-2} for laboratory-grown and 48 J cm^{-2} for outdoor-grown cultures). Interestingly, the decrease of C18 and C20 SFAs corresponded to the increase of C16:2, C16:4, C18:1, C18:2, C18:3 and C20:4 USFAs (Fig. 5; ESI Fig. 2, 3 and 9†). A similar result was obtained in UV-B-treated *Spirulina*, concomitant with deleterious effects on thylakoid membrane integrity and protein profiles.[42] Amongst USFAs, the increment of PUFAs was the main change in the present study. As PUFAs are involved in cell repair and growth,[43] it is conceivable that *Tetraselmis* cells attempted to repair the photo-damage caused by UV-C. However, photo-damage appeared irreversible at high UV-C doses since the lipid profile did not change in 24 h after 500 or 1000 mJ cm^{-2} exposure.

The fatty acid synthesis pathway of *Tetraselmis* sp. is considered to be similar to that of *Chlamydomonas reinhardtii*[44] where fatty acid desaturation results in insertion of double bonds into pre-formed fatty acid chains. Therefore, we speculate that the increases of C16:2, C16:4, C18:1, C18:2, C18:3 and C20:5 unsaturated fatty acids were actually the products of the desaturation of C16, C18 and C20 SFAs in *Tetraselmis* sp. M8 (ESI Fig. 10†). Low UV-C dosage may help to convert SFAs to unsaturated fatty acids with a significant increase in PUFAs. In microalgae, the deleterious effect of UV light on thylakoid membrane integrity leads to the production of reactive oxygen species (ROS)[42] (ESI Fig. 4†). PUFAs have a strong affinity or absorption to ROS;[45] therefore, the increment of PUFAs can be interpreted as a defence response against UV-C-generated cellular ROS.

The increase in USFA when compared to SFA in open raceway ponds confirmed the results obtained for lab-scale studies. This may have implications on biodiesel fuel properties that may possess better cold flow properties but may be more prone to oxidation. Further studies should be carried out to determine whether biosynthesis of nutraceuticals such as eicosapentaenoic acid (EPA) can be manipulated by UV-C radiation. A significant two-fold increase of EPA was detected on the laboratory scale (ESI Fig. 3†). UV-C treatment also significantly reduced co-cultured microorganisms (ESI Fig. 9†), which may be advantageous for water recycling and for minimizing contamination during continuous cultivation, an important factor in commercial production. Fig. 4a suggests that some lipids are consumed by microalgae after peak accumulation and during harvesting (with or without UV-C), but final UV-C treatment for settling may reduce lipid degradation as cell mortality increases. The protocol of UV-C lipid induction has been successfully applied to non-flagellate *Chlorella* sp. and *Nannochloropsis* sp., resulting in an increase of total lipid production. This was visualized by Nile red staining. ESI Fig. 11† shows the effect of UV-C treatment on three other microalgae (one flagellate: *Tetraselmis chui*, and two non-flagellate strains: *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2). To test whether the LIS protocol also resulted in enhanced settling for other flagellate microalgae, *Dunaniella salina* and *Tetraselmis chui* were subjected to UV-C treatment at different dosages. As shown in ESI Fig. 12,† UV-C treatment also resulted in effective settling for both flagellate microalgae.

In summary, this study used low exposure of UV-C radiation to induce lipids and harvest microalgae at the same time. Outdoor cultivation of *Tetraselmis* sp. M8 in open raceway modules showed that UV-C radiation could be a rapid and effective tool for LIS of flagellate microalgae. UV-C radiation could also control co-cultured microorganisms, facilitate water recycling and may assist in subsequent lipid extraction by pre-treating cells, all of which could potentially be well integrated in a microalgae biorefinery concept. UV-C-mediated LIS may provide an important step towards commercial microalgal biofuel production.

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Rapid induction of omega-3 fatty acids (EPA) in Nannochloropsis sp. by UV-C radiation

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Key Words:	Nannochloropsis, Microalga, Omega-3, UV-C

Rapid induction of omega-3 fatty acids (EPA) in *Nannochloropsis* sp. by UV-C radiation

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Abstract

Omega-3 fatty acids, such as eicosapentaenoic acid (EPA), provide substantial health benefits. As global fish stocks are declining and in some cases are contaminated with heavy metals, there is a need to find more sustainable land-based sources of these essential fatty acids. The oleaginous microalga *Nannochloropsis* sp. has been identified as a highly efficient producer of omega-3 fatty acids. In this study, we present a new process to rapidly induce biosynthesis of essential fatty acids, including EPA in *Nannochloropsis* sp. BR2. Short exposure to UV-C at a dose of 100 or 250 mJ/cm^2 led to a significant increase in total cellular lipid contents when compared to mock-treated controls. A low dosage of 100 mJ/cm^2 also led to a two-fold increase in total EPA content within 24 h that constituted 30% of total fatty acids and up to 12% of total dry weight at higher dosages. UV-C radiation may find uses as an easily applicable external inducer for large-scale production of omega-3 production from microalgae.

Keywords: EPA; microalgae; *Nannochloropsis*; omega-3 fatty acids; PUFA; UV-C

Introduction

Within the last decade microalgae have emerged as a potential source of renewable energy and nutraceutical products. Of particular interest are long chain polyunsaturated fatty acids (LC-PUFAs), including omega 3 and 6 (ω-3,6) fatty acids. Omega-3 and 6 fatty acids are essential components for the growth of higher eukaryotes (Ward and Singh, 2005; Adarme-Vega et al. 2012). Nutritionally, arachidonic acid (AA, 20:4, ω-6), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) are the most important fatty acids belonging to this group of bioactive compounds. AA and EPA are components of mammalian cell membranes and are also precursors of the eicosannids, including the prostaglandins, a family of biological effectors involved in inflammatory responses, blood pressure regulation, blood clotting and cell signaling (Kinsella et al. 1990). These compounds have been proven to help in neonatal retinal and brain development, as well as cardiovascular health and disease prevention (Carlson et al. 1993; Crawford 2000; Gill and Valivety 1997; Neuringer et al. 1988).

Current sources of ω-3,6 LC-PUFAs, EPA and DHA are marine fish oil (e.g. from mullet and krill), however global fish stocks have been in decline since the late 1980s (Worm et al. 2006). Moreover, traces of heavy metals have been found in marine fish, rendering these fish harmful to consumers (Bourdon et al. 2010). Fish oil is also not suitable for vegetarians and the odor makes it unattractive. Microalgae are the primary producers of these compounds. Their ability to grow under autotrophic, mixotrophic and heterotrophic culture conditions and on non-arable land with limited water recourses makes them a preferred choice for commercial production of ω-3,6 PUFAs, EPA and DHA. There is a wide range of lipid induction techniques in microalgae, such as the use of nutrient stress, including nitrogen and/or phosphorus starvation, light irradiation, pH or temperature change, and exposure to heavy metals or other chemicals (Sharma et al. 2012). Of all the microalgae studied, *Nannochloropsis* sp. are known to

produce high amounts of ω-3,6 LC-PUFAs, EPA (Huerlimann et al., 2010; Lim et al., 2012). For example, *Nannochloropsis oculata* cultivated under nitrogen limitation conditions and an increase in temperature from 20°C to 25°C resulted in an increase in total lipids by two fold increase (Converti et al. 2009). Moderated use of UV-A radiation for seven days could lead to an increased production of fatty acids in *Nannochloropsis* sp. (Forján et al. 2011) and in a study carried out by Srinivas and Ochs (2012) on *N. oculata* the effect of UV-A at different levels resulted in significantly increased lipid to chlorophyll ratio. Although these protocols highlight the possibility to increase lipid production based on laboratory research, there are still many concerns for their application, such as difficulties with culture maintenance, elongated cultivation periods/low lipid productivity, high cost for large-scale cultivation, oil extractability and potential environmental impacts. In most cases, it is also uncertain whether the induced lipid production during cultivation was derived from the scarification of algal growth or not. Therefore, there is a desire to develop an effective protocol to stimulate lipid production, more importantly LC-PUFA, while sustaining cell growth. A recent study on *Tetraselmis* sp. indicates that exposure to small dosage of UV-C 100-250 mJ/cm^2 radiation can result in increased LC-PUFA accumulation, especially C18:4. However, the actual production of ω-3 fatty acids was only 8 µg/mL culture. The study also highlighted the impact to low dosage of UV-C radiation on fatty acid biosynthesis pathway in converting saturated fatty acids (SFAs) into LC-PUFAs. Based on these results it was hypothesized that the biosynthesis of these LC-PUFAs can be further increased in *Nannochloropsis* sp. by low dosage of UV-C radiation, as naturally these species are known to produce high amounts of LC-PUFAs, especially EPA (Huerlimann et al., 2010).

Materials and methods

Laboratory-scale microalgae culturing and UV-C treatment

Nannochloropsis sp. BR2*,* family (Eustigmataceae) was originally collected from the Brisbane River and was cultured in the Algae Biotechnology Laboratory at The University of Queensland (Lim et al. 2012). Cultures were grown in f/2 medium (Guillard and Ryther 1962) in artificial sea water obtained from CSIRO at 23°C on an orbital shaker (Thermoline) at 100 rpm with 12 hours of light and dark rhythm. When the cultures were at late exponential growth phase and the cell densities reached $7.5x10^5$ cells/mL, cultures were subjected to UV-C radiation. The method for UV-C treatments has previously been reported (Sharma et al. 2014).

Lipid fluorescence analysis and GC-MS analyses

Nile red staining was conducted followed by flow cytometry analysis using a BD LSR II: Analyzing flow cytometer with 573 nm of excitation wavelength as described previously (Sharma et al., 2014). A total of 10,000 cells were counted in each sample. A gate was set to separate lipid producing fluorescence-activated cells and inactivated cells based on the analysis of cells without Nile red staining. The background absorbance from chloroplast auto-fluorescence was subtracted from each sample using mock-treated (control) *Nannochloropsis* sp. BR2 cultures that were not Nile red stained. Qualitative observation was conducted simultaneously by fluorescence microscopy as described below. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out as described previously (Sharma et al., 2014).

Dry weight measurements

A total of 5 mL of the culture were used for dry weight measurements. The culture was filtered through a 0.27 µm glass fiber filter (Millipore) which was pre-weighed and pre-washed with 1 mL distilled water

in a vacuum-subjected filter unit (three biological replicates were used from each culture (UV-C treated and mock-treated control)). After filtration, the filters were kept in individual Petri dishes to avoid contamination and dried in a drying oven for 24 h at 80°C with the plate lid half open, prior to weighing. To determine the dry weight the following formula was used:

$$\text{Dry weight mg/L} = \frac{\text{Filter dry weight - Filter pre-weight}}{\text{Filtered volume mL x 1000}}$$

Dry weight in mg/L was determined from the average of three weight measurements for each replicate.

Analytical methods

Data for growth rates and lipid productivities were statistically analyzed by one-way analysis of variance (ANOVA) with different microalgal cultures as the source of variance and growth rate or lipid productivity as dependent variables. This was followed by Turkey's multiple comparisons test (P>0.05) where appropriate.

Results

Low UV-C exposure leads to increased lipid accumulation in *Nannochloropsis* cells

Nannochloropsis sp. cells have previously been shown to accumulate significant amounts of lipids after nutrient deprivation and were identified as one of the most promising autotrophic microalgal sources for omega-3 fatty acid production (Huerlimann et al., 2010; Lim et al. 2012). Preliminary experiments

using combined stresses of nutrient starvation and UV-C radiation on *Tetraselmis* sp. have indicated that UV-C exposure can be an efficient stimulus to induce LC-PUFA production in microalgae, including EPA (Sharma et al. 2014). Thus in an effort to optimise EPA production in *Nannochloropsis*, UV-C treatment was applied to nutrient-starved *Nannochloropsis* sp. BR2 cells. Following Nile red staining, it became quickly apparent that lipid fluorescence intensities (yellow fluorescence) became stronger with an increase of UV-C radiation from 100 to 500 mJ/cm^2 (Fig. 1a). The reported cell rupturing that was observed in *Tetraselmis* sp. at 1000 mJ/cm^2 (Sharma et al., 2014) was not apparent in *Nannochloropsis* sp. BR2, suggesting that cell walls are quite rigid (Fig 1a). Based on the presence of green color in live cells and grey or brown color in dead cells, survival rates were determined by cell counting in a hemocytometer. This showed a gradual decline of live cells when UV-C radiation increased (Fig. 1b). The cell survival rates reached half (LD50) at UV-C radiations of 100 - 250 mJ/cm^2 and at 500 mJ/cm^2 and 1000 mJ/cm^2 only 5% of the cells had survived. There was no significant difference observed in the cell size at different UV-C radiation intensities, in contrary to reports for *Tetraselmis* sp. (Sharma et al. 2014). With an increase of UV-C radiation, the algal cells were divided into two different populations of P1 and P2, according to the fluorescence intensity (Fig 2 a-f). P1 population presented the auto-fluorescence of chloroplasts in the cells, corresponding to control cultures which were not stained by Nile red, while P2 represents Nile-red fluorescing cells (Fig 2a). In nutrient-starved but UV-C untreated control cultures, about 75% and 35% of the cells were present in P1 and P2 populations, respectively. Whereas, as the UV-C radiation increased from 100 to 1000 mJ/cm^2 a sharp decline was observed in the P1 population which was accompanied by sharp increase in the P2 population (Fig 2 a-f). P1 started to decline sharply with an increase of UV-C radiation. The P2 proportion increased significantly from 100 to 500 mJ/cm^2 (P<0.05) and was maintained at 95% between 500 and 1000 mJ/cm^2 (P>0.05) (Fig 3a). Overall an increase in Nile red-fluorescing P2 population and a decrease in unstained P1 population with an

increase in UV-C intensity was observed that were consistent with results previously obtained for *Tetraselmis* sp. (Sharma et al., 2014).

Based on the average fluorescence value, P1 cells displayed much less fluorescence intensity than P2 (Fig 3b). Different from P1, P2 cells had a big variation of fluorescence intensity that correlated to the total lipid intensity (Fig 3b). Compared to the mock-treated control cells, the lipid fluorescence intensity of algal cells was significantly increased with the increase in UV-C radiation. However, there was no significant difference between the mock-treated control (0 mJ/cm^2) and 100 mJ/cm^2 –treated cells (Fig 3c). As UV-C was increased to 250 mJ/cm ^{2} and 500 mJ/cm^2, the total lipid fluorescence nearly doubled when compared to the control (Fig 3c). Subsequently, the lipid intensity of P2 cells sharply dropped to the control level at 1000 mJ/cm^2 (Fig. 3b,c). However, the total lipid fluorescence was still increased significantly at 1000 mJ/cm^2 when compared to the mock-treated control (Fig 3c).

Fatty acid profiling in *Nannochloropsis* following UV-C radiation shows lipid induction and a shift towards LC-PUFAs

To further quantify the ability of UV-C stress to increase cellular fatty acid contents and to profile their composition, GC-MS analyses were carried out. These confirmed the results obtained from flow cytometry, showing a significant total fatty acid increase (P=0.002, P=0.031; respectively) of UV-C-treated cultures (100 and 250 mJ/cm^2) compared to mock-treated, nutrient-starved control cultures, whereas cultures treated with 500 and 1000 mJ/cm^2 showed no significant difference (Fig. 4a). Moreover, in cultures treated with 100 and 250 mJ/cm^2, the amount of unsaturated fatty acids (USFA) significantly increased and also the proportion of USFA compared to total fatty acids (Fig. 4a,c). Amongst the saturated fatty acids (SFA), the highest increase was observed for C16 (palmitic acid), followed by

C14 (Fig. 4b). On the other hand, C18 and C20 did not show any increase in cultures treated with 100 and 250 mJ/cm^2 UV-C, whereas in cultures treated with 500 and 1000 mJ/cm^2 there was no significant increase of any fatty acid (Supplementary Fig. 1 and 2). When comparing different USFA, cultures treated with 100 and 250 mJ/cm^2 UV-C showed significant increases for all detected USFA, most notably C20:5 (EPA) which accounted to be nearly 30% of the total fatty acid, and was increased highly significantly at 100 mJ/cm^2 (P=0.0075) followed by 250 mJ/cm^2 (P= 0.0308) (Fig. 4c). Other PUFAs were also significantly increased, namely C16:1, C18:1 cis+trans, and C20:4 (Supplementary Fig. 2). EPA contents in cellular dry weight was measured to determine suitability of *Nannochloropsis* for EPA production. As shown in Fig. 5, EPA constituted up to 12% of total dry weight at higher dosages, however a lower dose of UV-C resulting in a lower EPA content, maybe preferred to ensure high overall EPA productivity.

Discussion

Consistent with the previous study conducted on *Tetraselmis* sp. by Sharma et al. (2014), it was reported that the low dosage of UV-C radiation could also stimulate lipid biosynthesis in *Nannochloropsis* sp. and induce the production of PUFAs. A radiation of 100 and 250 mJ/cm^2 was optimal to induce total lipids, especially USFAs which were increased by two-fold compared to cells subjected to nutrient stress only, followed by SFAs (Fig. 4). A notable difference when comparing flow cytometry and GC-MC data was observed. The total fluorescence shown by flow cytometry analysis was significantly highest at 250 and 500 mJ/cm^2 followed by 1000 mJ/cm^2. Whereas, as per GC-MS analysis, maximum total fatty acid contents were measured following UV-C exposure at 100 and 250 mJ/cm^2. This difference in the lipid fluorescence can be attributed to the P2 population as the percentage of this population was higher in the culture treated at 250 mJ/cm^2 (75%) and 500 -1000 mJ/cm^2 (95%). Thus it was understood that the

high proportion of P2 population and the considerably high number of live cells resulted in high total lipid fluorescence. But when considering the fluorescence of individual P2 cells, the culture treated at 100 mJ/cm^2 displayed the highest fluorescence, followed by 250 mJ/cm^2 and 500 mJ/cm^2 which corresponds well to the results obtained by GC-MS. Moreover, when considering the dry weight measurements, as expected there was a linear decline in the total dry weight due to increased cell death at higher dosages (Fig. 5). But on the other hand, there was a significant increase in the percentage of cellular EPA with maximum values at 1000 mJ/cm^2 (up to 12% of total dry weight; Fig 5).

Cellular lipids may simply provide more energy reserves for stress responses. Alternatively, a UV-induced morphological variation maybe better adapted to adverse condition/lipid production. Since, UV-C causes genetic mutations (Guihéneuf et al. 2010; Rothschild 1999), UV may have acted as a selection pressure during algal evolution (Cockell 2000; Cockell and Raven 2007). In the previous study conducted on *Tetraselmis* sp. (Sharma et al. 2014) it was reported that at the higher dosage of UV-C (1000 mJ/cm^2) the release of lipid bodies became apparent as cell structures disintegrated, However cell rupturing was not observed in this study, the possible reason for this could be the relatively small cell size of the *Nannochloropsis* cells when compared to *Tetraselmis* sp. cells, also *Nannochloropsis* sp. cells reportedly possess rigid cell walls when compared to other microalgal species (Iqbal and Theegala 2013).

When comparing to other lipid induction techniques (Takagi et al. 2006; Wang et al. 2008; Sharma et al. 2012), UV-C radiation resulted in faster lipid biosynthesis stimulation within 24 h (Fig. 1-4). Of all the fatty acids measured, a significant increase was observed in the production of the omega-3 fatty acid EPA which was induced by 2.5-fold compared to control cultures that were subjected to nutrient stress only, and accounted to be approx. 30% of the total fatty acids (Fig 4c), followed by a two fold increase for C18:1, C18:4, C20:4 (Supplementary Fig. 2). The previous study reported that UV-C radiation on

Tetraselmis sp. lead to an increase in reactive oxygen species (ROS) production (Sharma et al., 2014). PUFAs have a strong affinity or absorption to ROS (Bouhamidi et al. 1998), therefore, the increment of PUFAs in this study can be interpreted as a defense response against UV-C-generated cellular ROS.

The role of PUFAs as an antioxidant and an agent to prevent cell damage has been well documented in many studies on plants and animals (Bartsch et al. 1999; Cheeseman and Slater 1993; Lenzi et al. 2000; Machlin and Bendich 1987). Omega-3 fatty acids (including EPA) have been found to play an important role in prevention of degenerative and cardiovascular diseases (Hooper et al. 2004; Ross et al. 2007). Moreover EPA has been reported to be part of the cell membrane of microalgae and to play an important role in cellular repair and membrane development in microalgae (Huerlimann et al. 2010; Valentine and Valentine 2004). Thus this can also be one of the reasons that *Nannochloropsis* sp. *cells* were still intact at higher UV-C dosage of 1000 mJ/cm^2 as cells had relatively high concentrations of EPA (Fig. 5) when compared to the previous study (Sharma et al., 2014).

In conclusion, this study highlights the use of low exposure of UV-C radiation to induce lipids in *Nannochloropsis* sp., particularly EPA, within 24 h of the treatment. This presents a significant improvement to lipid induction by nutrient starvation only. The optimized large-scale production of EPA in *Nannochloropsis* may provide a much-needed alternative, vegetarian and land-based source of omega-3.

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Figure legends

Figure 1: Nile red-stained cells of *Nannochloropsis* sp. BR2 that received different doses of UV-C exposure after nutrient starvation. **a)** Cells with maximum lipid fluorescence (yellow) can be observed at 250 mJ and 500 mJ UV-C radiation. Cells are shown at 40x magnification (bar=50 µm) at 24 h after treatment. **b)** Kill curve of *Nannochloropsis* sp. BR2, showing the number of cells that survived UV-C treatment at different dosages. Shown are mean values ± SEs from three independent treatments. The arrow indicates the LD50 value.

Figure 2: FACS analysis of *Nannochloropsis* sp. BR2 unstained cells (**a**), Nile red-stained mock-treated control cells (**b**), UV-C treated cells (c-f) at 100, 250, 500 and 1000 mJ/cm^2, respectively, showing P1 (background fluorescence) and P2 (Nile red fluorescence) populations. The Y-axis shows fluorescence intensity at phycoerythrin excitation wavelength of 575 nm and the X-axis shows the forward scatter based on cell size.

Figure 2: Lipid induction in *Nannochloropsis* sp. BR2 at 24 h after receiving different UV-C dosages. FACS analysis of Nile red-stained cells showing distribution (**a**) and lipid fluorescence (**b**) of low (P1) and high (P2) fluorescence cell populations and of the total population (**c**). Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with different letters indicate significant differences (P<0.05).

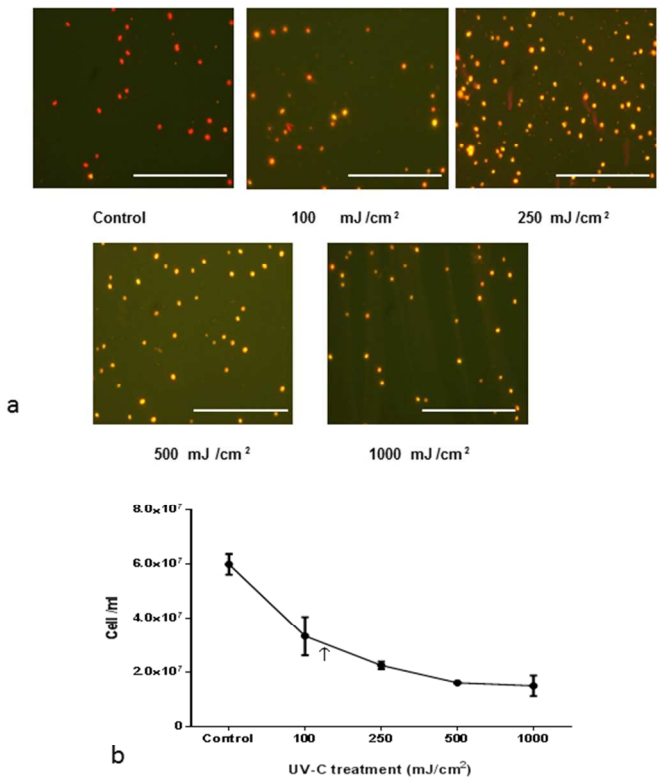
Figure 3: Fatty acid profiling and quantification by GC-MS showing (**a-c**) total fatty acids as well as saturated (SFA) and unsaturated (USFA) fatty acids, respectively, produced by different UV-C-treated *Nannochloropsis* sp. BR2 cultures. Shown are mean values ± SEs from three separately-grown and

treated microalgal cultures. Bars with (*) indicate significant differences (P<0.05) and bars with (**) indicate highly significant difference (P<0.01).

Figure 5: Dry weight and EPA proportion changes in *Nannochloropsis* sp. BR2, following nutrient starvation and different UV-C exposures. Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with different letters indicate significant differences (P<0.05).

Supplementary Figure 1: Comparison of different saturated fatty acids present in *Nannochloropsis* sp. BR2 following different treatments of UV-C radiation. Values are mean ±SE (n = 3); bars with different alphabets indicate significant differences (P<0.05).

Supplementary Figure 2: Comparison of different unsaturated fatty acids present in *Nannochloropsis* sp. BR2 following different treatments of UV-C radiation. Values are mean ±SE (n = 3); bars with different alphabets indicate significant differences (P<0.05).



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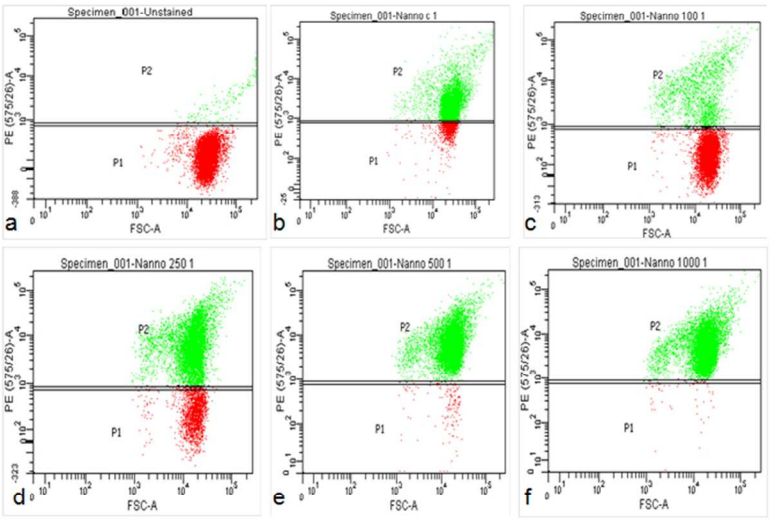

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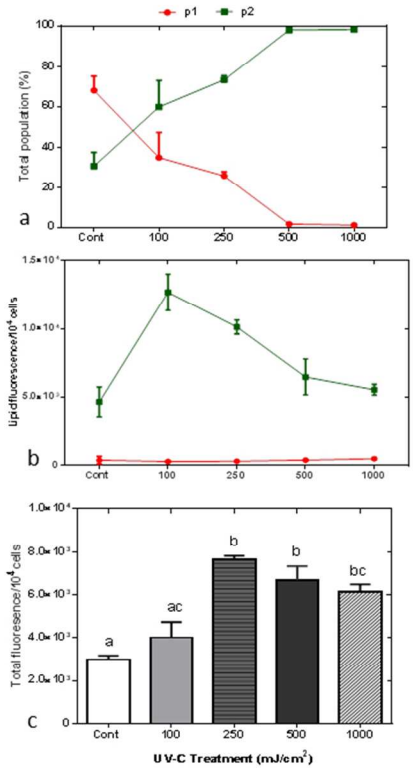

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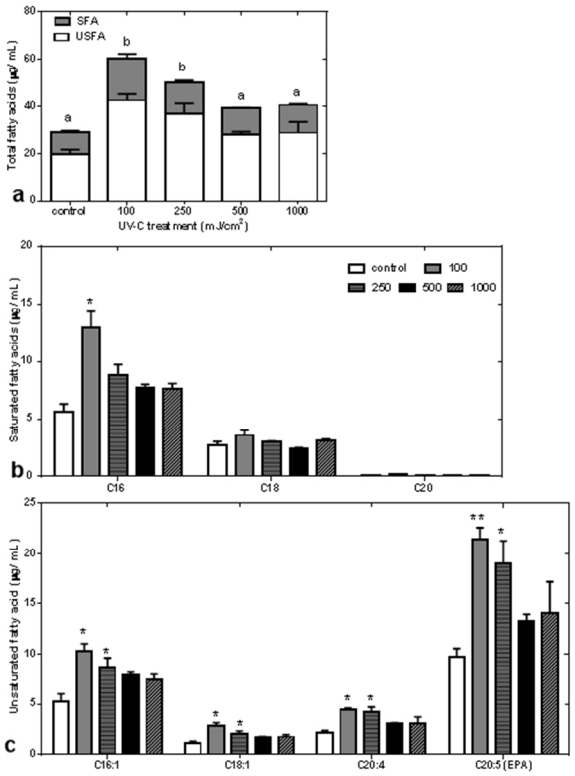


Figure 4: Fatty acid profiling and quantification by GC-MS showing (a-c) total fatty acids as well as saturated (SFA) and unsaturated (USFA) fatty acids, respectively, produced by different UV-C-treated Nannochloropsis sp. BR2 cultures. Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with (*) indicate significant differences ($P<0.05$) and bars with (**) indicate highly significant difference ($P<0.01$).
190x275mm (96 x 96 DPI)

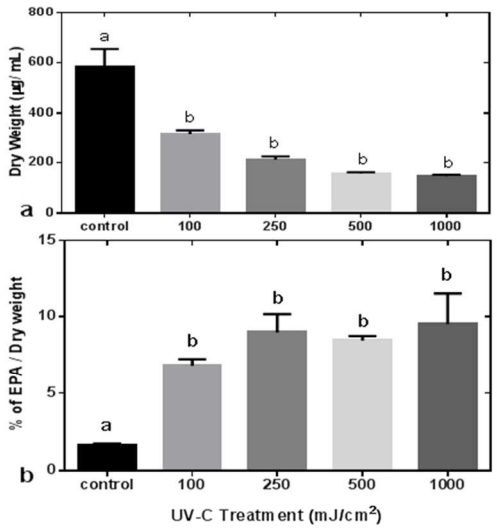
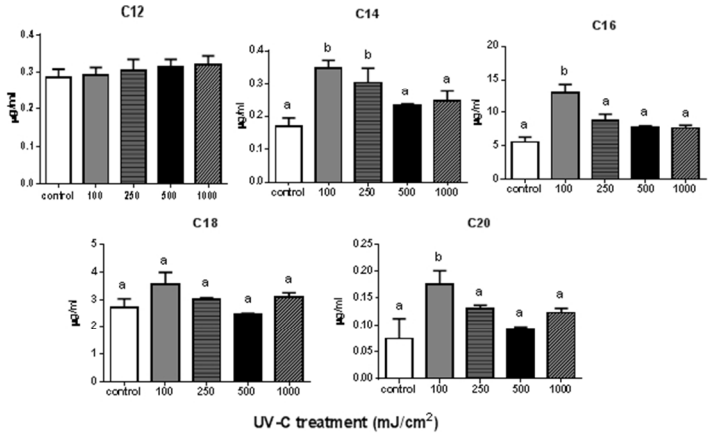
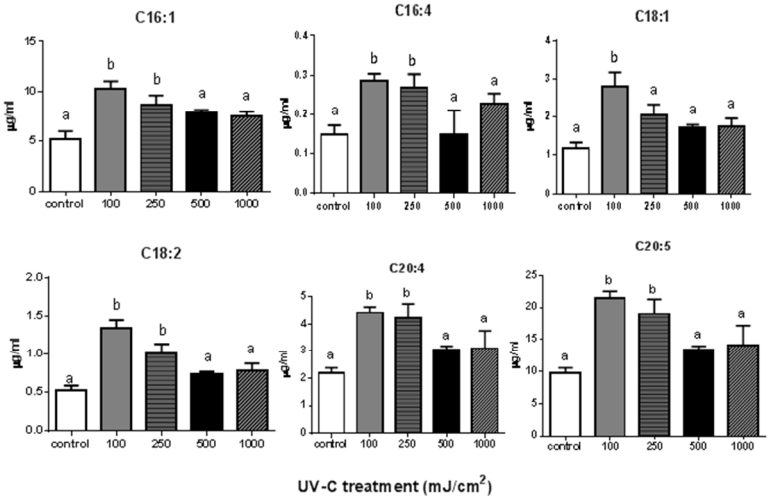


Figure 5: Dry weight and EPA proportion changes in Nannochloropsis sp. BR2, following nutrient starvation and different UV-C exposures. Shown are mean values ± SEs from three separately-grown and treated microalgal cultures. Bars with different letters indicate significant differences (P<0.05).
190x275mm (96 x 96 DPI)



Supplementary Figure 1: Comparison of different saturated fatty acids present in Nannochloropsis sp. BR2 following different treatments of UV-C radiation. Values are mean ±SE (n = 3); bars with different alphabets indicate significant differences (P<0.05).
190x275mm (96 x 96 DPI)



Supplementary Figure 2: Comparison of different unsaturated fatty acids present in Nannochloropsis sp. BR2 following different treatments of UV-C radiation. Values are mean ±SE (n = 3); bars with different alphabets indicate significant differences (P<0.05).
190x275mm (96 x 96 DPI)

BioEnergy Research

Rapid lipid induction in Chlorella sp. by UV-C radiation

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Rapid lipid induction in *Chlorella* sp. by UV-C radiation

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Abstract

Rapid induction of lipid accumulation in microalgae is an important prerequisite towards the use of microalgae as a feedstock for biodiesel production. In this study we present a novel approach to induce lipids in *Chlorella* sp. within 24 h by short term UV-C radiation (UVR) stress at different energy intensities ranging from 0-1000 mJ/cm^2. Increase in the lipid fluorescence was measured by Nile red staining and fluorescence-activated cell sorting analysis followed by gas chromatography-mass spectrometry. Lipid fluorescence was significantly increased in cultures radiated at or above 250 mJ/cm^2 compared to the mock-treated control cultures. Lower dosages at 100 mJ/cm^2 and 250 mJ/cm^2 led to a near doubling of total fatty acids, with a significant increase in total unsaturated fatty acids. This study provides a protocol for rapid lipid induction of microalgal cells by UV-C and the possible impact of UV-C radiation on fatty acid metabolism.

Keywords

Chlorella, Lipids, Microalgae, PUFA, UV-C

Introduction

Microalgae have become increasingly important feedstocks for different industrial processing applications like biofuel, high valve compounds and carbon sequestration [1,2]. The interest in microalgae for these potentials predominantly focuses on their high growth rates [3] and high lipid content, especially non-polar triacylglycerides (TAG) suitable for biodiesel production [4]. Being secondary metabolites, however, the production of TAGs is normally not compatible with the high growth rates in microalgae. Therefore, finding suitable methods to increase the lipid production without challenging algal growth rates is one of the major obstacles hindering the development of microalgae industrial application.

As microalgae have immense diversity, screening for optimal strains is the most important criterium for their potential utilization, particularly for uses in valuable oil or biofuel applications strains should display relatively high lipid contents, fast growth and easy cultivation and harvesting properties. *Chlorella* sp. is successfully cultivated at large-scale for commercial purposes and is considered a good feedstock for algal biodiesel production [5]. Hence, there are many attempts to increase lipid production/productivity in this species. For example, lipid content of *C. vulgaris* could be significantly increased by 40% in low nitrogen medium [6]. With manipulated culture conditions of 1.0 mM KNO_3, 1.0% CO_2, 60 µmol photon $m^{-2}s^{-1}$ and 25^{o}C, the lipid production of *C. vulgaris* was further increased 2.5-fold [7]. In addition, the lipid stimulation in *Chlorella* sp. was also achieved via silicon deficiency [8] and iron supplementation [9]. Similar to these autotrophic culture conditions, the yield of bio-oil from heterotrophically-grown *C. protothecoides* increased 3.4 times and made up to 58% of dry weight [10-12]. When co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*, about 4-fold lipid production was obtained in *C. vulgaris* [13]. Although these protocols highlight the possibility to increase lipid production based on laboratory research, there are still many concerns for their promotion and application, such as the difficulty of maintenance, elongated cultivation periods leading to low lipid productivity, high cost for large-scale cultivation and potential environmental impacts. In most cases, it is also uncertain whether the induced lipid production during cultivation was derived from sacrificing of algal biomass growth or not. Therefore, there is a need to develop an effective technique to rapidly stimulate lipid production, while sustaining high cell growth.

Different from the conventional cultivation protocols that aim at biomass, accumulation of lipids in microalgae has been achieved mainly under unfavorable and stressful conditions [14-16]. Due to the disturbance on normal metabolism of membrane phospholipids during rapid cell division, stress can induce cessation of cell division and diversion of photosynthetic energy into TAG production [15]. In addition to nutrient starvations, stresses also include temperature and pH changes as well as high

irradiance (e.g. UV-light) [17,15]. In comparison, UV radiation (UVR) is a sound protocol for microalgae lipid induction in large-scale cultivation. As UV radiation has genetically and physiologically deleterious effects on many life forms including microalgae [18], the impact is conceivably related to the radiation intensity. A study conducted by Forján et al. showed that the modulated use of UV-A radiation for seven days could lead to increased in production of fatty acids in *Nannochloropsis* sp. [19].

Previous UVR research in microalgae was mainly focused on the impact of UV-A and UV-B radiations on algal growth, morphological and physiological changes and triggered oxidative stress [19-25]. Compared to UV-A and UV-B, UV-C has more energetic radiation which is believed to have more adverse impacts on microalgae. UV-C has recently been applied to co-stimulate microalgal lipid production in *Tetraselmis* sp. together with nutrient stress at the end of its growth phase, resulting in an increase in long chain-polyunsaturated fatty acids (LC-PUFA), especially C18:4, including a two-fold increase of ω-3 fatty acids [26]. Therefore, to find a rapid lipid induction system for microalgae, we investigated application of UV-C stress to microalgae whenever the biomass/growth is optimal during nutrient replete conditions, rather than at the end of the growth phase.

In the current study, we used *Chlorella* sp. exposed to instant UV-C radiation as a module to study the efficiency of UV-C radiation on algal lipid stimulation. The variation of lipid production was quantified by fluorescence intensity changes in the cells by Nile red staining. Changes in individual fatty acid composition were further quantified by GC-MS analysis, demonstrating a clear and rapid significant induction at a dose of 250 mJ/cm^2 UV-C. In the meantime, the deleterious impact on algal cells was also measured by cell survival rate and chlorophyll pigments quantification.

Materials and methods

Microalgae cultivation and UV-C treatment

Chlorella sp. BR2 culture was originally collected from the Brisbane River (GPS coordinates South -27° 31' 21.36" East +153° 0' 32.87") and was cultured in the Algae Biotechnology Laboratory at The University of Queensland as previously described [27]. Primary stock cultures were maintained aerobically in 100 ml Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent white light (120 μmol photons m−2s−1). For experiments, *Chlorella* sp. BR2 was grown in f/2 medium [28] in autoclaved tap water. The culturing conditions were set at 23°C on an orbital shaker (Thermoline) at 100 rpm with 12 hours of light and dark rhythm. When the cell density reached 1.6x10^7/mL, which is still in the late exponential growth phase for this strain [26], the culture was used for UV-C radiation trials. After gently stirring, 5 mL aliquots of *Chlorella* sp. BR2

culture were pipetted into a Petri dish, forming a thin layer inside (total of 20 plates). Plates were randomly divided into five groups with three plates used for each for UV-C radiation (253 nm) treatment in a UV chamber (Biorad, Gs-Genelinker). They were separately irradiated at 0, 100, 250, 500 and 1000 mJ/cm^2 as described previously [26]. All Petri dish cultures were subsequently incubated for 24 h and algae survival rates were measured by counting the live cells based on visibly intact chloroplasts in each replicate. The cell size was also measured by compound microscopy (Olympus). One of four Petri dish cultures was randomly selected and used for thin layer chromatography (TLC) analysis as suggested by Timmins et al, [29]. Briefly, all cells were centrifuged at 4000xg for 5 min and dried at 70°C overnight. The dried algal cells were grinded and dissolved in chloroform, methanol and water (v/v/v; 1:2:0.8). The extraction was filtered (0.22 um) and chloroform and water (v/v; 2:1.8) were added for phase separation. Then, the lipid-containing supernatant layer was transferred into an Eppendorff tube and the chloroform was evaporated in a desiccator. While 20 µL chloroform were added to resuspend the extracted lipid, 3 µL were loaded on a 10 x 10 cm HPLC-HL silica gel plate (Anatech) (n=3). The mobile phase consisted of n-hexane, diethyl ether and acetic acid (v/v/v; 80:20:2).

Lipid fluorescence analysis and GC-MS analysis

For each replicate, 1 mL of algae cells was sampled and stained with Nile Red solution and used for fluorescence-activated cell sorting (FACS) analysis as described previously [26]. For each sample, the fluorescence of 10,000 cells was quantified and a gate was set up to distinguish fluorescence-activated cells and inactivated cells based on the analysis of cells without Nile red staining [26]. The background absorbance from chloroplast auto-fluorescence was subtracted from each sample using mock-treated (control) *Chlorella* sp. BR-2 culture. Qualitative surveillance of stained cells was carried out simultaneously by fluorescence microscopy as described previously [26].

GC-MS analysis was carried out as described previously [26] using 4 mL of algal culture per sample.

Analytical methods

Data for lipid productivity was statistically analysed by one-way analysis of variance (ANOVA) using lipid productivity as dependent variables and different microalgal cultures as the source of variance.. This was succeeded by Turkey's multiple comparisons test ($P<0.05$) where appropriate.

Results

UV-C–irradiation stimulates microalgal lipid accumulation but increases cell mortality

To test whether rapid lipid induction could be achieved on actively dividing cells, *Chlorella* sp. BR2 cultures during exponential growth phase were treated with various doses of UV-C radiation. As expected for rapidly dividing cells, lipid yellow fluorescence after Nile red staining was rarely observed among the cells in the control cultures while lipid fluorescence intensities were increasingly stronger with higher of UV-C radiation doses (Fig. 1a). At higher doses, cell rupture was also observed starting from 500 mJ/cm^2 and became more prominent at 1000 mJ/cm^2, as shown by the presence of free small yellow lipid bodies released into the medium (Fig. 1a). Results obtained from TLC also confirmed the Nile red observations, where a bright band of TAGs was visible for cultures treated at 100 and 250 mJ/cm^2 (Online Resource Fig. 1). As expected the algal survival rate decreased gradually with increasing UV-C radiation (Fig. 1b). Cell survival rates reached half at a UV-C radiation of 100 mJ/cm^2 (LD50), and at 500 mJ/cm^2 to 1000 mJ/cm^2 only 5% of the cells had survived, respectively. By contrast, the cell size gradually became larger with an increase of UV-C radiation (P<0.05, Fig. 1c).

UV-C induces lipid fluorescence in *Chlorella* populations

To profile and quantify lipid accumulation in *Chlorella* cells following different UV-C exposure, a flow cytometer was used (Online Resource Fig. 2). With an increase of UV-C radiation, algal cells were divided into two different populations, named P1 and P2, according to a background fluorescence intensity cutoff in the unstained control population. The majority of the P1 population in this control was attributed to auto-fluorescence of chloroplasts (Fig. 2a). In the untreated, but Nile red-stained control and in the culture treated with 100 mJ/cm^2 UV-C, 99% of the cells were attributed to the P1 population, whereas only 1% were present in the P2 population (Fig. 2a,b). When UV-C radiation of 250 mJ/cm^2 was used for treatment, P1 was reduced to 25%, while the lipid-accumulating P2 increased to 74%, indicating a marked increase in lipid production after UV-C treatment (Fig. 2a). However, at 500 and 1000 mJ/cm^2 only 3% of the cells were present in the P1 population and nearly all (97%) cells were present in the P2 population. The P2 population started to split into another two clusters at UV-C radiations of 250 mJ/cm^2 and 500 mJ/cm^2 (Online Resource Fig. 2). However, this tendency was not apparent at 1000 mJ/cm^2 where only one cell cluster was observed.

Quantification based on average fluorescence intensities from three separately-grown and -treated microalgal populations, showed that P1 cells always had much lower fluorescence intensities than the lipid-accumulating P2 populations (Fig. 2b). Compared to the mock-treated control, the lipid fluorescence

intensity of algal cell was significantly increased with an increase in UV-C radiation. However, there was no significant difference between the control and the 100 mJ/cm^2 treatment (Fig. 2b,c). When UV-C radiation was applied at 250 and 500 mJ/cm^2, the total lipid fluorescence nearly increased by 30 and 70 times respectively. Subsequently, the lipid intensity of P2 cell sharply dropped to the control level at 1000 mJ/cm^2 (Fig. 2c). However, the total fluorescence was increased nearly 20 times at 1000 mJ/cm^2 when compared to control. It should be mentioned that this strong increase in lipid fluorescence was probably influenced by the presence of free lipid bodies at higher UV-C dosages (Fig. 1a) and not a direct measure of cellular lipid accumulation.

UV-C treatment induces production of polyunsaturated fatty acids (PUFA)

To further quantify fatty acid contents and profile individual fatty acids, GC-MS was carried out on algal cultures. The results obtained from GC-MS analyses confirmed the results obtained by flow cytometry, showing a significant increase in total fatty acid in UV-C-treated cultures (100 and 250 mJ/cm^2) compared to untreated controls, whereas cultures treated with 500 and 1000 mJ/cm^2 showed no significant difference (Fig. 3). Amongst the saturated fatty acids (SFA), the highest increase was observed for C16 (palmitic acid), followed by C20 and C14 (Online Resource Fig. 3). On the other hand, C12 (lauric acid) did not show any increase in cultures treated with 100 and 250 mJ/cm^2 UV-C, whereas in cultures treated with 500 mJ/cm^2 the amount of C18 was significantly higher when compared to the mock-treated control cultures. When comparing different unsaturated fatty acids (USFA), cultures treated with 100 and 250 mJ/cm^2 UV-C showed significant increases for all detected USFA, most notably C16:1, C16:2, C18:1 , C18:3 and C20:4 (Online Resource Fig. 4).

Discussion

This study firstly introduced UV-C radiation to induce lipids production in *Chlorella* sp. concomitant with lipid induction, the algal cells were also relatively altered. While the cell size gradually increased with higher UV-C dosages, the cell survival rate significantly declined. Out of the different UV-C intensities tested, the maximum lipid fluorescence was displayed by cultures radiated at 500 mJ/cm^2 (Fig. 2c). However, the presence of free lipid bodies from ruptured cells at higher UV-C dosages (Fig. 1a) rather than intracellular lipids probably contributed to this. This is also suggested by the TAG data obtained from TLC (Online Resource Fig. 1) and GC-MS data on total fatty acids (Fig. 3) where UV-C dosages of 100 and 250 mJ/cm^2 led to the highest induction compared to the untreated control cells. GC-MS data further revealed that the UV-C-based lipid induction led to both, an increase in saturated and unsaturated fatty acids, especially at 100 and 250 mJ/cm^2. (Fig. 3), although to a different extend for

individual fatty acids (Online Resource Fig. 3 and 4). This study highlights the efficiency of UV-C radiation on rapid microalgae lipid induction on actively-dividing *Chlorella* cells, and also provides a benchmark for rapid lipid analysis by FACS of microalgae cells by UV-C. Compared to lipid induction by conventional cultivation and stress stimulations, e.g. by gradual nutrient depletion [9,37], UV-C radiation only take a few seconds or minutes and lipid stimulation could be obtained within 24 hours. It should be investigated whether UV-C radiation may serve as a viable alternative to achieve higher lipid productivities in large-scale cultivation systems where cells can remain in exponential growth phase, in comparison to cells that have to undergo lengthy nutrient depletion phases.

Coincident with many studies conducted with UV-B radiation [e.g. 30,31], an increase in cell size was also found in this study. Similarly, a study on nitrogen deprivation in the marine microalga *Dunaliella tertiolecta* also noted an increase in cell size with more lipid accumulation [32]. So it was implied that the cell size may have increased by UV-C as a result of lipid induction. However, larger cells could be better adapted to tolerate UV radiation as suggested by previous UV-B research [30,31]. Since UV can cause genetic mutations on most life forms [18,33], UV may have acted as a selection pressure during algal evolution [34,35].

Along with the increase of cell size, the cell survival rate declined dramatically in this study and some of the cells had burst open and lipid bodies were released in medium (Fig. 1a). This emphasizes the need to carefully optimize UV-C dosages to achieve maximum lipid induction (and lipid productivity) for the culture. However, a pre-treatment with UV-C that leads to breaking of the cells may have a positive effect on lipid extraction efficiency.

In this study, the P1 population in FACS analyses presented the auto-fluorescence of algal cells, while P2 was attributed to lipid fluorescence (Online Resource Fig. 2). The conversion of cells from P1 to P2 was a clear sign of lipid stimulation by UV-C radiation. The lipid stimulation in *Chlorella* sp. BR2 was closely correlated to the amount of P2 population between 100 and 500 mJ/cm^2, where the average lipid content in P2 cells also increased significantly (Fig. 2). Although the total lipid content increased by UV-C radiation at different extents, an alteration of fatty acid profiles was significant at 100 and 250 mJ/cm^2 (Fig. 3; Online Resource Fig. 3 and 4). Interestingly, the decrease of C16, C18 and C20 SFAs corresponded to the increase of C16:2, C16:3, C18:1, C18:2, C18:3 and C20:4 USFAs (Fig. 4). A similar result was also obtained for *Spirulina platensis* with UV-B radiation, concomitant with deleterious effect on thylakoid membrane integrity and protein profile [36].

Amongst these USFAs, the increment of PUFAs was the main change in this study. As PUFAs are involved in cell repair and growth [38,39], it is conceivable that *Chlorella* sp. BR2 cells were repairing the photo-damage caused by UV-C. However, it seems that the damage was irreversible when algal cells were exposed to high UV-C radiation since the lipid profile did not change in 24 hours at 500 and 1000 mJ/cm^2 of UV-C radiation (Online Resource Fig. 3 and 4)

Fatty acid desaturation inserts double bond(s) into pre-formed fatty acid chains [40]. Therefore, it is tempting to speculate that the increase of C16:2, C16:3, C18:1, C18:2, C18:3 and C20:4 USFAs were actually the products of the desaturation of C16, C18 and C20 SFAs in *Chlorella* sp. BR2 (Fig 4). Thus it can be concluded that lower UV-C dosage (i.e. 100 and 250 mJ/cm^2) may help to convert SFAs to USFAs with a significant increase in PUFAs within 24 h after treatment.

In microalgae, the deleterious effect of UV light on thylakoid membrane integrity and protein profile is accompanied by the generation of reactive oxygen species (ROS) [36,41]. It has been proven that PUFAs have a strong affinity or absorption to ROS [41]. Therefore, the increment of PUFAs may present a protection mechanism of algal cells to ROS, while membrane damage due to lipid peroxidation would also create the need to repair membrane damage with polar lipids containing PUFAs.

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Figure legends

Figure 1 a) Nile red-stained *Chlorella* sp. BR2 cells observed at 40x magnification with a scale size of 50 µm. Cells with maximum lipid fluorescence (shown as yellow) were observed at 250 and 500 mJ/cm^2 UV-C radiation. As the radiation was increased to 1000 mJ/cm^2 cell rupturing was apparent (white arrow) with lipid bodies released into the medium. **b**) Killing curve of *Chlorella* sp. BR2, showing the number of cells that survived treatment with UV-C at certain UV-C radiation doses. Values are mean ± standard error (n = 3). **c**) Cell size of *Chlorella* sp. BR2 after different UV-C treatments. Values are mean ± SE from three separately grown cultures (n = 3). Different letters indicate significance differences (P<0.05).

Figure 2: FACS analysis of *Chlorella* sp. BR2 at 24 h after receiving different UV-C dosages. (**a**) cells present in P1 and P2 population (**b**) Lipid fluorescence of P1 and P2 population (**c**) Total fluorescence of 10,000 cells at different UV-C dosage. Values are mean ± SE from three separately-grown cultures (n = 3). Bars with different letters indicate significant differences (P<0.05).

Figure 3: Total fatty acids produced by different UV-C-treated *Chlorella* sp. BR2 cultures. Values are mean ± SE from three separately grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).

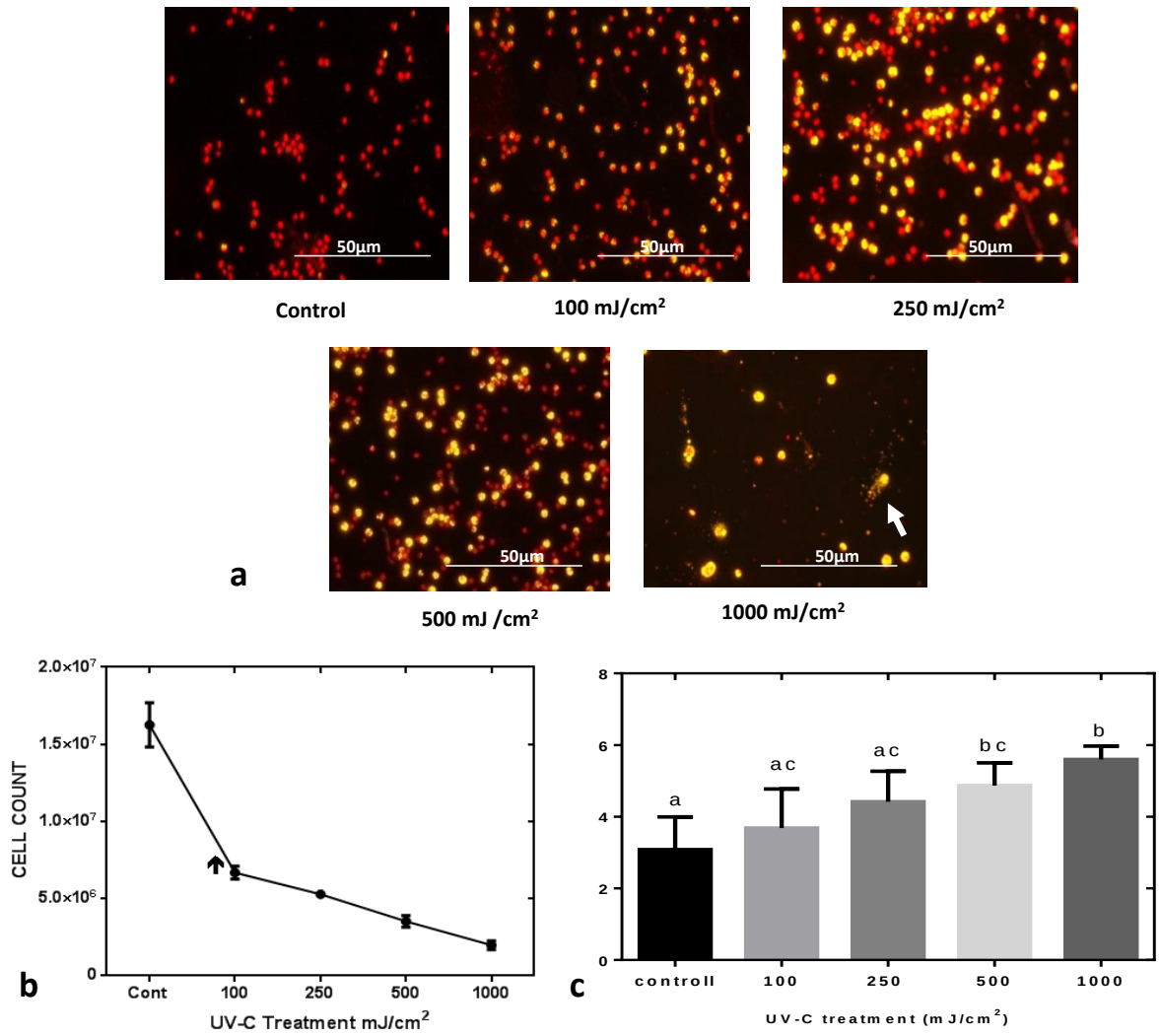
Figure 4: Effect of low dosage of UV-C radiation on the fatty acid synthesis pathway. Energy obtained from UV-C radiation might help in conversion of saturated fatty acids that serve as a storage function in plastids to unsaturated fatty acids that serve as antioxidants and repair damage to membrane lipids.

Online Resource Figure 1: Analysis of the non-polar fraction of TAGs in C*hlorella* sp. BR2 treated with different UV-C radiation doses (0-1000 mJ/cm^2).

Online Resource Figure 2: FACS analysis of *Chlorella* sp. BR2. Shown are cells without Nile red staining (Unstained Cells) and Nile red-stained with different UV-C dosages ranging from 0 mJ/cm^2 (Control) to 1000 mJ/cm^2 showing P1 and P2 populations. The Y-axis shows fluorescence intensity at the phycoerythrin excitation wavelength of 575 nm and the X-axis shows the forward scatter based on cell size.

Online Resource Figure 3: Comparison of different saturated fatty acids present in *Chlorella* sp. BR2 cultures treated with different doses of UV-C radiation. Values are mean ± SE from three separately-grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).

Online Resource Figure 4: Comparison of different unsaturated fatty acids present in *Chlorella* sp. BR2 cultures treated with different doses of UV-C radiation. Values are mean ± SE from three separately-grown cultures (n = 3); bars with different letters indicate significant differences (P<0.05).



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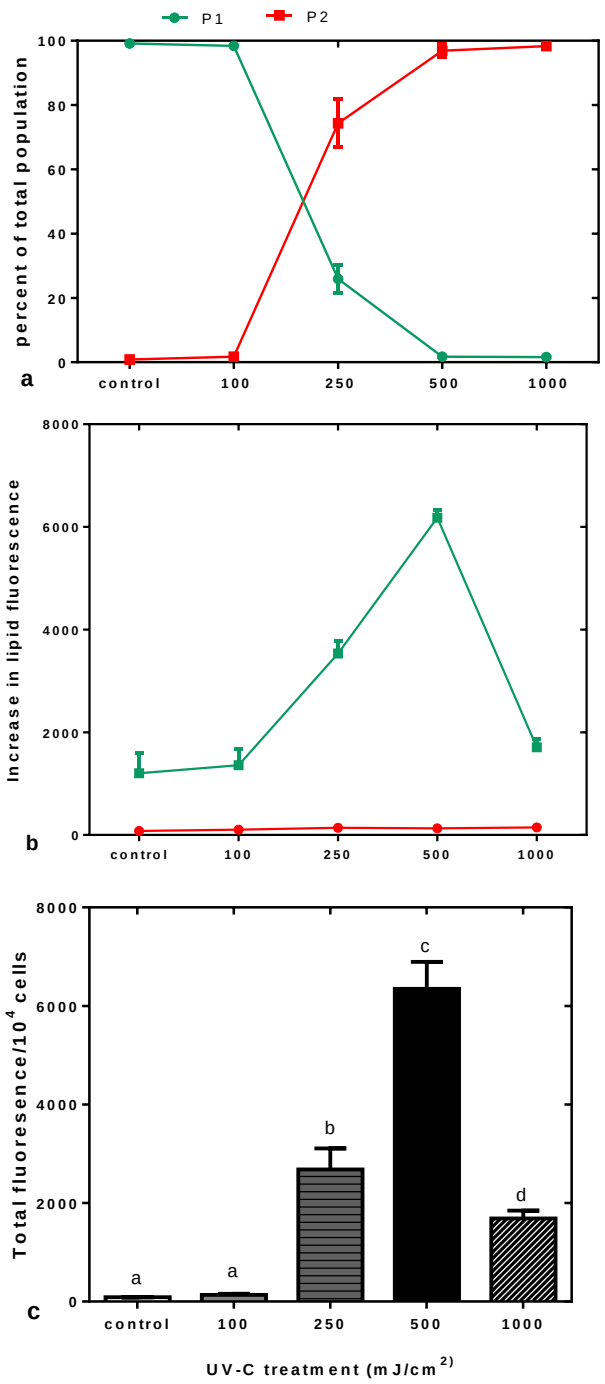

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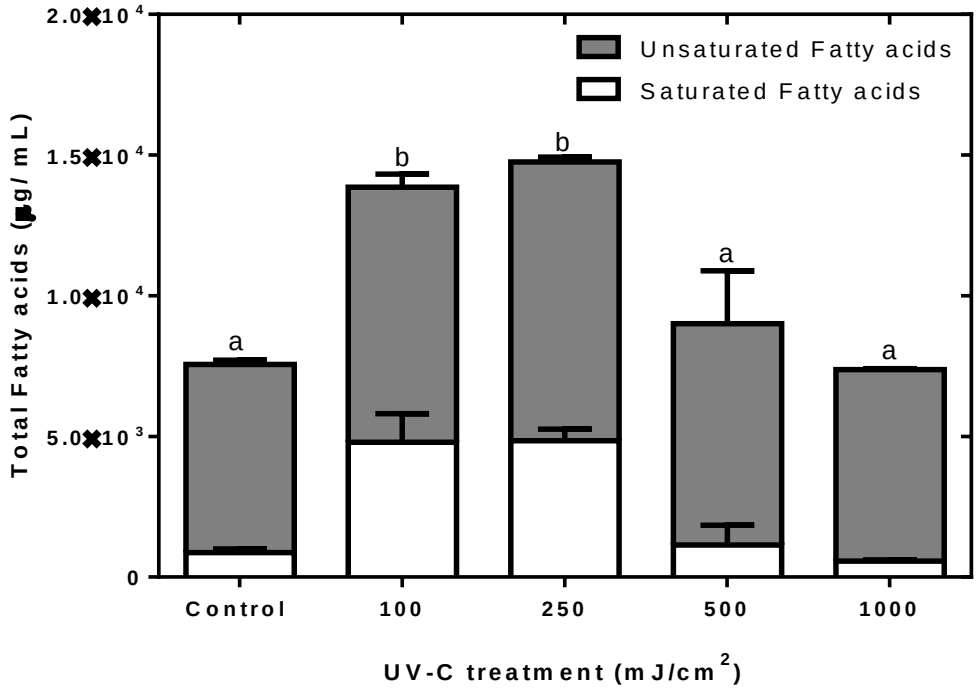

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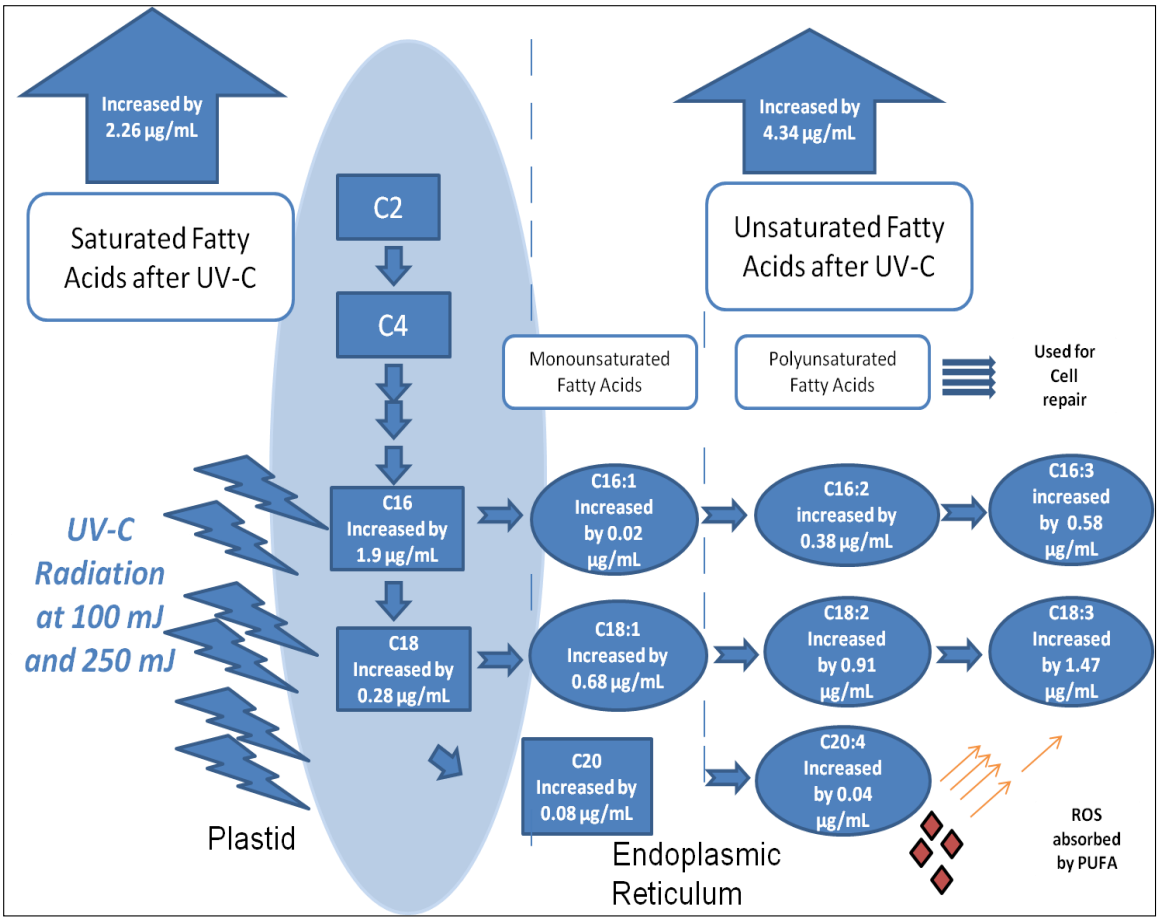

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Supplementary Material

Click here to download Supplementary Material: Online Resource Figure 1.docx

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BioEnergy Research

Isolation of high-lipid Tetraselmis suecica strains following repeated UV-C mutagenesis, FACS and high-throughput growth selection

--Manuscript Draft--

Manuscript Number:	BERE-D-14-00218R2
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Abstract:	Mutagenesis and selection of microalgae can be used for accelerated breeding of elite strains, providing a significant advantage over genetic engineering as prior biochemical and genetic information is not required. UV-C-induced mutagenesis combined with fluorescence-activated cell sorting (FACS) and microtiter plate reader cell density screening was used to produce Tetraselmis suecica strains with increased lipid contents without compromising on cell growth. After five rounds of mutation-selection, two dosages of UV-C (50% and >98% lethality) yielded two improved strains (M5 and M24) that produced significantly more neutral lipids (increases of 114% and 123%, respectively). This study highlights that repeated UV-C mutagenesis and high-throughput selection for cell growth can be a viable combined approach to improve lipid productivity in microalgae. These maybe used as elite strains for future breeding programs and as potential feedstock for biodiesel production.

Editor's comments:

Reviewer 1 is happy with the revisions and has no further comments. Reviewer 2 still has some remaining requests for revisions, and I am concerned that these are somewhat unfair, especially the comment about the starch, which you indicated would be part of a follow-up study.

My suggestion is that you make a number of minor edits to the text, and include a statement about the need to investigate the relationship between starch and TAG, and plans to investigate this further, but that you do not conduct any further experiments. Please let me know if that sounds like an acceptable solution.

Authors' response:

We agree that altered carbon partitioning in the selected strains could explain the increase in lipids and we have therefore added the following sentence to the Discussion (Line 477):

"Future studies should also investigate whether carbon partitioning is altered in the selected strains and whether the increased lipid content may result from a decrease of starch reserves as was found for other strains with elevated lipid contents [42]."

Reviewer 1:

I think Manuscript Number BERE-D-14-00218 should be accepted to be published in "BioEnergy research" now since the authors have answered the questions decently.

Reviewer 2:

The authors provide answers to my comments. However, I am not convinced about the fact that the mutants do not present higher lipid content under N replete conditions by looking at Figure 5, which shows at t0 a more elevated fluorescence for M5, with a very

large standard deviation, from only 2 independent replicates (by the way usually 3 biological replicates are used). So in my mind, this point needs to be clarified by experimental data (change deplete by replete in lines 417-418 of p 13).

Authors' comments:

We agree that it is possible that lipid content in the selected strains could also slightly be elevated during nutrient replete conditions. However, no statistically significant differences could be found (even in earlier studies that were not part of this time course experiment). The mistake was corrected (Line 416).

Reviewer 2:

In addition, in my mind, it would be relevant to measure at least starch content, to obtain a clear view of the changes occurring in the mutants. Indeed, many papers (for example de Jaeger et al 2014, Yu et al 2013, Work et al 2010) report about decreased starch content when increased TAG levels and it seems for me a rather elementary point to be known at that stage of the research.

Authors' response:

Please see Response to Editor above.

Reviewer 2:

Additional comment

If I understand well, figure 6 only shows relative values of the different fatty acids. I imagine that it would also be possible to give total FA content per cell (in pg) from the information got by GC-MS. This value would be very useful because none of the other figures give absolute lipid quantification.

Authors' response:

The GC-MS data was analysed to determine absolute values and the

following sentence was added to the manuscript (Line 445):

“GC-MS data only showed a slight increase of total fatty acid contents in the selected strains (30.7 and 29.7 µg/mL for M5 and M24, respectively) compared to the wt (26.8 µg/mL), raising the question whether lipids other than fatty acids may have contributed to the higher lipid fluorescence in the selected strains.”

Other changes include the addition of another author and a new citation [42].

Isolation of high-lipid *Tetraselmis suecica* strains following repeated UV-C mutagenesis, FACS and high-throughput growth selection

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Highlights

- UV-C mutagenesis and high throughput selection technology has been combined to improve lipid productivity in *Tetraselmis suecica*.
- Both 50% and >98% lethal dosage successfully produced improved strains with approximately 100% increase in lipid accumulation.
- Growth rates of improved strains remained unchanged.

Abstract

Mutagenesis and selection of microalgae can be used for accelerated breeding of elite strains, providing a significant advantage over genetic engineering as prior biochemical and genetic information is not required. UV-C-induced mutagenesis combined with fluorescence-activated cell sorting (FACS) and microtiter plate reader cell density screening was used to produce *Tetraselmis suecica* strains with increased lipid contents without compromising on cell growth. After five rounds of mutation-selection, two dosages of UV-C (50% and >98% lethality) yielded two improved strains (M5 and M24) that produced significantly more neutral lipids (increases of 114% and 123%, respectively). This study highlights that repeated UV-C mutagenesis and high-throughput selection for cell growth can be a viable combined approach to improve lipid productivity in microalgae. These maybe used as elite strains for future breeding programs and as potential feedstock for biodiesel production.

Introduction

As fossil fuel resources diminish and cause environmental damage, there is a rapidly-growing global demand for lipids, particularly triacylglycerides (TAGs) for the biofuel, aquaculture and pharmaceutical industry. The need to develop sustainable lipid sources is now widely apparent. Traditional crop-based plants are increasingly being used for oil and biofuel production, but these cannot reasonably meet the growing demand [1]. Photosynthetic microalgae have repeatedly been proposed as a more viable lipid source due to their high productivity, environmental benefits and ability to produce different kinds of oils [2-4]. Theoretically, microalgae can produce 10 to 20 times more lipids than oil palms [5], corn and soybean [6-8] while achieving CO_2 capture efficiencies of up to 99% [9]. Furthermore, the production of microalgal biomass can be carried out without competing for valuable resources, such as arable land, biodiverse landscapes (e.g. rainforests) and freshwater [5]. However, the industry is still in its infancy and the cost of microalgal lipid production is still too high to achieve full commercialization of microalgal lipid feedstocks. Having the ideal algal strain with elevated lipid content, high growth rate and robust environmental tolerance remains one of the most important factors to improve algae economics [10,11]. While many studies have focused on species selection and characterization to identify strains that contain two seemingly antagonistic traits: fast growth and high lipid content, genetic and metabolic engineering provide opportunities to create potential elite strains that meet these requirements. At present, induced mutagenesis provides a significant advantage over genetic engineering, as little biochemical or genetic information regarding the chosen organisms is needed [12,13]. This approach requires relatively little technical manipulation. Improved non-transgenic microorganisms can be bred by incorporating mutagenesis and high-throughput selection, including microalgal strains with enhanced lipid performance.

Mutation studies involving ultraviolet (UV), ethyl methane sulfonate (EMS) and nitrosomethylguanidine (NTG) have been performed on a range of microalgal species (*Phaeodactylum tricornutum*, *Pavlova lutheri*, *Nannochloropsis oculata*,

Haematococcus pluvialis, *Schizochytrium* sp., *Chlorella sorokiana*, *Scenedesmus obliquus, Isochrysis galbana, Dunaliella salina*) to produce mutants that exhibited increased/modified lipid content and growth rate [14-23]. Many of these studies, however, rely upon time-consuming techniques like metabolite antibiotic response to select for mutants and lipid extraction techniques to analyze their lipid content. Lately, the combination of lipophilic dyes (e.g. Nile red) and high-throughput technologies such as microplate readers and flow cytometry provided a powerful tool to isolate potential mutants from a complex population based on specific fluorescence cell properties. A study using the Nile red fluorochrome in conjunction with microplate reader technology has been shown to be able to select *C. sorokiniana* and *S. obliquus* mutants with elevated lipid contents [21]. Automated fluorescence-assisted cell sorting (FACS) can be more efficient than a microplate reader-based selection, as much larger populations of single cells can be handled, resulting in the recovery of a high number of candidate cells with the desired lipid content. The use of FACS in combination with mutagenesis has been described in mutation-selection studies that produced high-lipid content strains of *I. galbana* [23] and carotenoid-hyperproducing *D. salina* strains [22]. Even without mutagenesis, the use of FACS has been successful in generating higher lipid content strains of *Nannochloropsis* sp. and *T. suecica* through selection alone [1,24]. While most studies involve only one screening step, the present study combines a mutation-selection approach using UV-C-induced mutagenesis [19-21,23] with FACS [1,24] and microplate reader screening [21] to mutate, identify and isolate *T. suecica* cells with a higher lipid content without reduced growth rate.

Tetraselmis suecica is a flagellate green microalga commonly used as aquaculture feedstock, and that is also considered a good candidate for biofuel production. This species is known to have a high lipid content as well as being robust enough to tolerate a range of environmental conditions [25,26]. Moreover, *Tetraselmis* cells have recently been shown to lose their flagella during stressful conditions, quickly settling and thus reducing harvesting/dewatering cost [27]. The growth characteristics of the *T. suecica* strain, used in the present study, have been previously described, displaying one of the highest comparative microalgal

growth rates, although with a slightly lower TAG content compared to other strains tested [28]. Therefore, this microalga was chosen as a suitable candidate to generate an improved strain with fast growth and high-lipid content properties.

Material and Methods

Microalgae culturing conditions

The parent culture of wild-type *Tetraselmis suecica* (wt) was originally collected by Queensland Sea Scallops (Bundaberg, Australia; [28]) and cultured in autoclaved artificial seawater with Guillard F medium [29] and maintained aerobically in 100 mL Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent white light (120 µmol photons $m^{-2}s^{-1}$).

Mutagenesis

The mutation program used UV-C (100-280 nm) as the mutagenic agent. First, the appropriate dosage of UV-C treatment was determined in order to obtain an equivalent of the rate of non-lethal mutations. To achieve this, kill curves were produced by two methods, to establish 50% (LD50) and >98% lethal dosage.

Mutagenesis was achieved by placing a 2 cm-deep culture of *T. suecica* (1 x 10^6 cells/mL; early starvation phase) in a 140 mm-Petri dish under the lamps of a Bio-Rad GS Gene Linker UV Chamber and exposure to 15, 25, 50 and 100 mJ of UV-C. Cultures were then left in the dark for 24 h to prevent DNA repair by photo-reactivation. The first kill curve was obtained by plating 200 µL aliquots of 1/1000-diluted mutagenized cells onto a 1% agar plate containing F medium in artificial seawater, which were then allowed to grow for 3 weeks before algal colonies from control and UV-exposed cells were counted. The second kill curve was carried out in 96 well-plates inoculated with mutagenized cells after serial

dilution down to 1 cell per well [22]. Wells with surviving microalgae that multiplied were then counted after 3 weeks of growth. Both, agar plates and 96 well-plates were grown under fluorescent white lights (50 µmol photons $m^{-2}s^{-1}$; 16:8 h light:dark photoperiod) and maintained at 24°C. Further stages of this study used UV-C dosages of 25 mJ and 100 mJ to induce mutagenesis, as these provided a survival rate of 50% and <2%, respectively.

FACS and lipid quantification by Nile red fluorescence

Nile red (Sigma Inc.) was used to stain lipids for (i) FACS and (ii) quantification of lipid contents via a 96 well-microplate reader. First, the appropriate amount of Nile red working solution required to produce the best lipid staining, while maintaining a high FACS survival rate, had to be determined. Cell samples in starvation phase (1 mL at 0.8-1 x 10^6 cells/mL) were treated with 1, 2 and 3 µL of a working solution of Nile red in acetone or dimethyl sulfoxide (both 1 mg/mL). Samples were then gently mixed and incubated in the dark for 10 min. Single cells were sorted using a BD FACSVantage SE (Becton Dickinson) cell sorter with a 485 nm argon laser and 100 µm nozzle into 96 well-plates using F in seawater medium. Cell fluorescence was measured at 585 nm for yellow-gold fluorescence, indicative of neutral lipid content. Approximately 10,000 cells were analyzed, with dot plots of yellow-gold fluorescence (PE-A) vs. forward light scatter (FSC-A, cell size). Cell sorting regions were positioned to include cells presenting increased fluorescence and size compared to the general population of cells. FACS survival rates were then determined after 2 weeks of growth.

To enable quantification of neutral lipid contents of *T. suecica* cells in a microtiter plate reader, the ability of Nile red (in acetone working solution) to stain neutral lipids without killing the cells was first established. Two populations of microalgal cells (10^6 cells/mL): (i) cells in late starvation phase and (ii) cells in exponential growth phase were mixed to produce a population of 0, 25, 50, 75 and 100% of starved cells, as a proxy for increasing lipid content within a given volume. A total of 1 mL of these samples was then stained with 2

μL of a working solution of Nile red in acetone (1 mg/mL). Samples were then gently mixed and incubated in the dark for 10 min. A total of 100 μL from each sample was then loaded into a 96 well-microtiter plate (Sarsted) in triplicates. Yellow-gold fluorescence was measured on a POLARstar OPTIMA (BMG Labtech) plate reader using fluorescence intensity mode. Gain was set at 3000, with excitation and emission wavelengths of 485 nm and 590 nm, respectively. These settings were used for further fluorescence intensity measurements in this study.

Mutation-selection cycles

Cultures were grown until late log phase and then mutagenized according to the optimized method at 25 mJ and 100 mJ. Mutagenized cultures were then left in the dark for 24 h and then cultured for 2 weeks to allow for culture recovery (addition of fresh F medium every 7 days). Two days prior to FACS, nutrient deprivation to stimulate lipid production was achieved by removal of previous medium by centrifugation (1,200 x g, 5 min) and replacement with only seawater (without F medium). FACS was then carried out according to the optimized methodology, with mutagenized single cells sorted into 96 well-plates. Plates were incubated at 24°C under a 16:8 h light:dark photoperiod of fluorescent white lights (50 μmol photons $m^{-2}s^{-1}$). For generations (cycles of mutagenesis and selection rounds) 1, 2 and 3, the plates after FACS were monitored daily, and the first 6 wells that showed visual signs of algal growth were selected and scaled up for subsequent rounds of mutagenesis and cell sorting. For generations 4 and 5, the top 24 wells from the 96 well-plates after FACS that showed visual signs of growth were scaled up (1:10 dilution) in a 24 well-plate. After 7 days of growth at 24°C under a 16:8 h light:dark photoperiod of fluorescent white light (50 μmol photons $m^{-2}s^{-1}$), the absorbance value (450 nm) and fluorescence intensity of the wells were measured. Cell counts were performed on the top 12 wells that showed the highest fluorescence/absorbance ratio using a haemocytometer. The top 3 clones that showed the highest fluorescence/cell count ratio were selected and scaled up for subsequent rounds of mutagenesis. In all cycles of the program, selected strains were allowed to

grow for 3 to 4 weeks to ensure a genetically stable population before mutagenesis.

Standard protocol for culture growth analysis, lipid induction phase, sampling for fluorescence and lipid analysis

A standard protocol was designed to allow direct comparison of growth rates, fluorescence intensity and fatty acid (FA) profile between selected strains and wt based on a modified method by Lim et al. [28]. Briefly, a total of 5 mL of selected strain or wt culture in late log phase was used as inoculum (8 h after start of the light cycle) for 50 mL artificial seawater complemented with F medium in 100 mL Erlenmeyer flasks, and grown under constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light:dark photoperiod of fluorescent white light (120 µmol photons $m^{-2}s^{-1}$). After day 7, nutrient deprivation to stimulate lipid production was achieved by centrifugation (1200 x g, 5 min) and replacement with only seawater (without F medium). Cultures were then grown for another 5 days post starvation. Cell counts were performed on days 0, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12 & 13, while fluorescence intensities were measured on days 0, 2, 3, 4, 5, 6 post starvation. The fatty acid methyl ester (FAME) contents of the cultures were analyzed on day 4 post starvation.

Fatty Acid Methyl Ester (FAME) analysis

For FAME analyses, 4 mL of algal culture was collected from each replicate and centrifuged at 16,000 x g for 3 min. After the supernatant was discarded, lipids in the algal pellet were hydrolyzed and methyl-esterified by shaking (1,200 rpm) with 300 µL of a 2% H_2SO_4/methanol solution for 2 h at 80°C. Prior to the reaction, 50 mg of heneicosanoic acid (Sigma, USA) was added as internal standard to the pellet. A total of 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane was then added followed by mixing for 20 s. Subsequently, phase separation was performed by centrifugation at 16,000 x g for 3 min. A total of 1 mL of the hexane layer was then injected splitless into an Agilent 6890 Gas Chromatograph coupled to a 5975 MSD Mass Spectrometer. A DB-Wax column

(Agilent, 122–7032) was used with running conditions as described for Agilent's RTL DBWax method (Application note: 5988–5871EN). Quantification of FAMEs was carried out by taking the ratio of the integral of each FAME's total ion current peak to that of the internal standard (50 mg), with the molecular mass of each FAME also factored into the equation. FAMEs were then identified based on mass spectral profiles, in comparison to standards and expected retention times from Agilent's RTL DBWax method (Application note: 5988–5871EN).

Analytical methods

Growth rates and doubling times were calculated from day 0 to day 7 to measure the growth rate during growth phase, day 0 to day 10 to measure the overall growth rate, and from day 7 to day 10 to measure the growth rate during starvation phase.

Calculations for growth rate and doubling time are based on the following equations:

Growth rate $\mu = \mathrm{Ln}(Ny/Nx)/(ty-tx)$

Mean doubling time $TAve = (ty-tx)/\log2\,(Ny/Nx)$

with Ny and Nx being the number of cells from the selected days of analysis.

Results

Mutagenesis survival rate

The appropriate dosage of UV-C exposure to obtain a 50% and <2% survival rate was determined by treating microalgal cells with a range of UV-C dosages (0-100 mJ), followed by growth on agar- (solid media) and in 96 well-plates (liquid media). Survival rate (Figure 2a) was found to be dosage-dependent, with the survival rate decreasing as UV-C dosage increased. The

growth method was also had an effect of post-UV-C exposure survival rates. When grown on agar plates, the LD50 was found to be at 16 mJ, while <2% survival rate was found to be at 63 mJ. In liquid medium the 50% survival rate and <2% survival rate was at 26 mJ and 92 mJ, respectively (Figure 2a) and this dosage was used during the subsequent experiments.

Optimization of Nile red staining for Fluorescence-Activated Cell Sorting (FACS)

The use of appropriate Nile red staining solution was required to ensure maximum fluorescence while maintaining a high recovery of viable clones. While the increase in staining solution did not affect fluorescence intensities or clone recovery, it was found that cells stained with Nile red dissolved in acetone produced fluorescence intensities markedly higher than cells stained with Nile red in DMSO (Figure 3). Unstained populations achieved a recovery rate of 78% viable cells, while both DMSO and acetone solvents achieved 40-50% recovery. Therefore for this study, the addition of 1 µL Nile red in acetone working solution was chosen for FACS. The ability of Nile red staining in acetone to determine varying levels of neutral lipids in *T. suecica* was also established (Figure 4). This study also found that 2 mg/mL of Nile red was suitable to detect populations of *T. suecica* with >12.5% of cells containing neutral lipids, demonstrating a strong linear correlation (r^2= 0.98, n=6) between the percentage of starved cells and fluorescence intensity.

Growth rates and cell density

After the 5th cycle of mutation-selection, two strains with one of the best fluorescence/cell count ratio (one from each UV dosage), were chosen for further analyses : (i) M5 (originating from 25 mJ UV-C (50% survival rate) mutagenesis) and (ii) M24 (100 mJ UV-C (<2% survival rate)) were compared with wt *T. suecica* to determine their growth and lipid production performance. During the first 7 days of growth, all cultures exhibited similar growth rates and cell densities. After starvation was induced, both M5 (0.2 day^{-1}; *P*=0.06) and M24 (0.2

day^{-1}; P<0.05) exhibited higher growth rates than wt. The strains also achieved significantly higher (P<0.05) cell densities than wt (1.25 x 10^6 cells/mL) on day 10, and reached 1.82 x 10^6 cells/mL and 1.71 x 10^6 cells/mL, respectively. Overall growth rates ($\mu_{10\ days}$) of the selected strains were also found to be slightly higher than wt, although not at a significant rate (Figure 5).

Neutral lipid content

After 7 days of growth, nutrient deplete conditions were used to induce lipid production, and the neutral lipid content of selected *T. suecica* strains and wt were determined. The total neutral lipid content of wt increased until day 3 and then plateaued, while the total neutral lipid content of M5 and M24 continued to increase. From day 4 onwards, total neutral lipid production of the selected strains was significantly higher than wt (P<0.05), with M5 and M24 achieving maximum total neutral lipids on day 5 (114% increase from wt) and day 6 (123% increase from wt), respectively (Figure 5a). On a per cell basis, wt cells accumulated lipids from day 2 to day 3 and then stopped, while selected strains cells continued to accumulate lipids until day 6. M5 cells had 80-90% more neutral lipids than wt on day 4 and 5 (P<0.05), while M24 cells contained 96%-100% more neutral lipids than wt on day 5 and 6 (P<0.05; Figure 5b).

GC/MS analyses revealed an overall reduction in monounsaturated FAs (MUFAs; P<0.05) in the selected strains on day 5 (Figure 6). This trend was observed for C16, C16:1, C18, C18:1, C20 and C20:1 FAs, where significant reductions were accompanied by significant increases in polyunsaturated FAs (PUFAs), such as C16:4, C18:4 and C20:5. The selected strains also exhibited an increase in C14 and a decrease in C18:2, although this was only significant (P<0.05) in the M24 strain.

Discussion

This study describes a combined and repeated mutation-selection method designed to increase the neutral lipid content of *T. suecica* without compromising

its growth rate. It also includes the optimization of key steps within the mutation-selection cycle, such as the appropriate UV-C dosage and Nile red concentration. UV-C has been successfully used to generate microalgae mutants [19, 21], and was selected as a mutagenic agent because it was practical and safer than chemical mutagens such as EMS and MTG. As chemical mutagens are more effective than UV radiation, UV-C mutagenesis requires higher dosages (with lower survival rates) [30]. However, although the frequency of mutants increases among survivors at high dosages, so does damage to the genetic background which reduces clone recovery and fitness. Therefore, two different UV-C dosages were chosen to either increase mutation probability (>98% lethal dosage) or increase recovery of a high number of clones (50% lethal dosage) with less genetic background damage, a survival rating similarly applied in other microalgae mutation studies [19,22]. The recovery of UV-C-exposed cells in liquid media was more effective, as survival rates in 96 well-plates were higher compared to solid media agar plates. The poor growth of other flagellate microalgae on solid media was also demonstrated for *Pavlova lutheri* as well as other filamentous and flagellate organisms, primarily due to the dehydration of the medium [31,32].

The use of Nile red as a fluorescence probe for neutral lipid detection and quantification in microalgae has been well documented [1,21,22,24,23,33,34]; a strong correlation between lipid content and fluorescence intensity has already been established [34]. The addition of solvents such as acetone and DMSO has been shown to improve the transition of the dye into lipids, although their efficacy varies between species, depending on the characteristics of the individual algae species [34]. The optimization stage of this study revealed acetone as a better stain carrier than DMSO for staining *T. suecica* cells that displayed higher fluorescence intensities during FACS. Other flow cytometry studies involving *D. salina* [33], *Nannochloropsis* sp. [1] and *I. galbana* [23] also utilized acetone as a carrier. The percentage of viable cells post-sorting achieved in this study (40-50%) was lower than the 80% reported by Montero et al. [24], which was achieved by seawater as sheath fluid, but still higher than the 20-30% reported by other studies involving the sorting of other phytoplankton

flagellates [35]. The staining efficacy of DMSO has been shown to be better than acetone at a higher volume/volume [34], but would prove toxic and reduce FACS clone recovery. Therefore when at similar volumes, acetone produced a higher fluorescence intensity than DMSO and was the preferred solvent in this study. This study has also established a correlation between Nile red fluorescence intensity between percentage of starved cell (a proxy of total lipid content within a volume). In *T. suecica*, correlations between Nile red fluorescence signal and TAG content [24], as well as neutral lipids estimated by gravimetry, have been established [36]. Other studies have also found a strong correlation between fluorescence signal and total lipid content [34,37], thus confirming the use of Nile red fluorescence to quantify lipid content in this study to allow for sorting of hyperlipidic *T. suecica* strains.

The overall aim of the mutation-selection program was to develop a strain that had a high lipid content while maintaining its high growth rate. For generations 1 to 3, lipid selection was achieved during flow cytometry, while growth rate selection was carried out post-sorting during the grow-up phase of the sorted cells. While other studies pooled their sorted cells [1,24], individual cells were sorted into individual 96 well-plate wells to facilitate the selection of the top six fastest growing individuals that would be carried into the next cycle of selection. The growth screening step was introduced to maintain the growth rate of the selected strains, as there have been studies that indicated reduced growth rates in strains isolated for high lipid content [22,8,38,21]. To confirm that the selected individuals still maintained their high lipid content, an additional screening step similar to that of Vigeolas et al. [21] was introduced in generations 4 and 5. A total of 24 instead of six of the fastest growing strains were selected and scaled up in 24 well-plates before their Nile red fluorescence intensity and absorbance values were measured to obtain fluorescence/absorbance. As absorbance values do not account for cell viability and can be misleading, cell numbers were then used to confirm the top six performers with the highest fluorescence/absorbance. The final mutation-selection program therefore now incorporates FACS with a fast growth selection step, followed by a high lipid per cell step.

After five cycles of mutation-selection that yielded two improved strains: M5 and M24, a standard protocol to compare the growth rates, lipid content and FA content was performed. Growth rate comparisons found the overall growth rates of improved strains to be slightly higher than wt, with significant increases occurring during the starvation period. Improved strains also achieved a significantly higher maximum cell density compared to wt. While the growth rates achieved in this study were expected to be lower than reported by Montero et al. [24] due to the lack of CO_2 aeration, FACS-isolated *T. suecica* cells in that study exhibited lower growth rates than the original wt. Reduced growth and cell density were also reported in a mutant study involving another flagellate, *D. salina,* that did not incorporate a growth selection step [22], although no reduction in growth rates was reported for *I. galbana* after two rounds of mutation-selection [23]. Other mutation studies that reported maintained or increased growth rates in mutants were found to have incorporated a growth selection step as well [15-17,21].

When comparing neutral lipid productivities, selected *T. suecica* cultures in the current study exhibited a 114-123% increase in total fluorescence compared to wt, and a 90%-100% increase on a fluorescence per cell basis (but the cultures did not show any significant differences during nutrient rdeplete conditions on day 0; Figure 5). These results were much higher than other UV mutation studies without FACS or Nile red fluorescence screening, which reported only a 8-35% increase in FA content [14,18,19]. Nevertheless, the yield improvement of this study was more similar to studies involving microplate reader screening and FACS. Vigeolas et al. [21] screened UV-mutated cells based on Nile red fluorescence using a 96 well-plate reader to develop *C. sorokiana* and *S. obliquus* strains with 50-300% increase in fluorescence units per cell, corresponding to similar increases in TAG content per cell, while Bougaran et al. [23] combined UV mutagenesis and FACS in an *I. galbana* mutation-selection procedure that increased lipid productivity by 80%. It is interesting to note that in FACS studies without mutagenesis, a *T. suecica* strain with up to 4-fold increase in fluorescence signal was obtained after two rounds of sorting [24], while a

Nannochloropsis sp. strain with a 3-fold increase in total lipid content was selected after three rounds of sorting [17]. While significantly higher than wt, the neutral lipid content of both improved *T. suecica* strains in the present study was not significantly different from each other (Figure 5). This indicates that both 50% and >98% lethal dosage can be used to produce viable mutants with selectable traits. Nevertheless, a 50% lethal dosage was preferred as the recovery of clones was easier and less time-consuming.

As cell sizes between selected cells and wt cells were not significantly different during FACS analyses, it is expected that, similar to a per cell basis (Figure 5b), lipid contents per dry weight would also be increased (although this was not directly measured here). Although selected cells were not found to be significantly smaller, it appears that both, a more rapid growth after N depletion (Figure 4) as well as an increased cellular lipid fluorescence (Figure 5b), contributed to an increased overall lipid fluorescence in the selected cells (Figure 5a). This is in alignment with the selection protocol (Figure 1) that selected for both of these traits. GC-MS data only showed a slight increase of total fatty acid contents in the selected strains (30.7 and 29.7 µg/mL for M5 and M24, respectively) compared to the wt (26.8 µg/mL), raising the question whether lipids other than fatty acids may have contributed to the higher lipid fluorescence in the selected strains. The comparison of FA profiles between improved strains and wt *T. suecica* revealed a decrease in MUFAs such C16:1 and C18:1, accompanied by increases in PUFAs such as C16:4, C18:4 and C20:5 (Figure 6). While certain studies report unaltered FA composition in their mutants [1,17,21], there have been studies that have also reported elevated PUFAs. The increase of PUFA in mutants has been documented in *P. lutheri* mutants irradiated by UV [19] and *Nannochloropsis* sp. irradiated by EMS and *N*-methyl-*N*-nitrosourea [15,16]. Furthermore, Chaturvedi & Fujita (2006) and Chaturvedi et al. (2004) also reported increases in C14 and reductions in C18:1. This decrease in the proportion of saturated FAs and MUFAs in comparison to an increase in PUFAs suggests that the mutants preferentially store lipids in the form of PUFAs. This, along with an overall increase in neutral lipids detected by elevated Nile red fluorescence, point towards mutations occurring in the gene

coding for ACCase enzyme and the coding sequences of key desaturases(s) genes. Changes in the ACCase enzyme, considered to be the rate-limiting step in FA biosynthesis [39], would increase the substrate pool of TAG production, leading to more short-chain FAs (e.g. C12, C14, C16) that become precursors for MUFAs and PUFAs, as well as an increase in overall TAG accumulation [16]. Variations in PUFA contents between wt and selected strains also point towards mutations in key desaturase genes, which could explain the shift towards PUFA production [16]. Another explanation towards increased PUFA production in selected strains is the antioxidant effect of PUFAs against reactive oxygen species (ROS) generated during mutagenesis [40]. The antioxidant function of PUFAs in marine microorganisms, particularly eicosapentaenoic acid (EPA), has been reviewed and points towards their stabilizing function against oxidation by ROS. This is achieved by increased PUFA presence as membrane phospholipids to function as shield molecules [41]. Therefore, during the course of multiple rounds of mutation and selection, selected strains with increased PUFA production would have increased survivability towards UV-C radiation. Future studies should also investigate whether carbon partitioning is altered in the selected strains and whether the increased lipid content may result from a decrease of starch reserves as was found for other strains with elevated lipid contents [42].

Considering the average doubling time of 2.55 days of the selected strains, the fluorescence values obtained during the comparison to wt were obtained more than 24 cell divisions after the final selection cycle step, and 36 divisions after UV radiation in cycle 5. This supports the idea of a stable genetic makeup of the obtained putative mutants that produce increased neutral lipid content. Although the nature of these putative mutations are beyond the scope of this study, the observed improvements could be a result of adaptation (e.g. by epigenetics), and not DNA mutations. It cannot be ruled out that strains adapted to, instead of mutated to UV-C exposure. Therefore, the lipid content of these strains should be evaluated again in the future, particularly after long-term storage without selection pressure for high lipid content. At that point, unchanged lipid content values in comparison to wt would indicate a stable genetic mutation, while epigenetic change would cause these strains to adapt

back a more wt phenotype. Unlike plant crops that undergo very few reproductive cycles between harvests (typically just one), bred elite microorganisms are constantly at high risk to revert back to faster growing wt-like strains. At present, the risks of this occurring for the microalgae in this study appear reduced, as the selected strains did not compromise on their ability to grow.

Acknowledgements

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Figure Legends

Figure 1. Mutation-selection cycle for the production of *T. suecica* cells for improved lipid production. Cycles 1-3: the top 6 clones with the fastest growth after each sorting run were selected for subsequent mutagenesis. Cycle 4 & 5: the top 24 fastest growers were scaled up and their lipid production performance evaluated. The top 3 lipid producing cultures were selected for subsequent cycles.

Figure 2. Optimization of UV-C lethal dosage and Nile red staining. (a) Survival rate of *T. suecica* in agar plates and 96 well-plates after exposure to varying UV-C dosages. The 10 mJ survival rates in 96 well-plates were not measured. (b) Fluorescence units of mixed starved & unstarved *T. suecica* population demonstrating the ability of Nile red staining in acetone to determine varying levels of neutral lipids in *T. suecica.* Data represent mean ± SEM from three independent replicates.

Figure 3. Two-dimensional dot plots of *T. suecica* stained with varying volumes of Nile red in acetone or DMSO working solution: (a) 1 µL Nile red-acetone, (b) 2 µL Nile red-acetone, (c) 1 µL Nile red-DMSO, (d) 2 µL Nile red-DMSO. Selected region of population P1 is an example of what was gated for cell sorting.

Figure 4. Cell density of *T. suecica* selected strains and wild-type over the span of 13 days with induced starvation on day 7. Boxed data points indicate significant differences from wild-type ($P<0.05$). Inserted table: Growth rates and doubling time for the first 7 days, first 10 days and from day 7 to day 10. Asterisks indicate significant differences from wild-type ($P<0.05$). Data represent mean ± SEM from two independent replicates.

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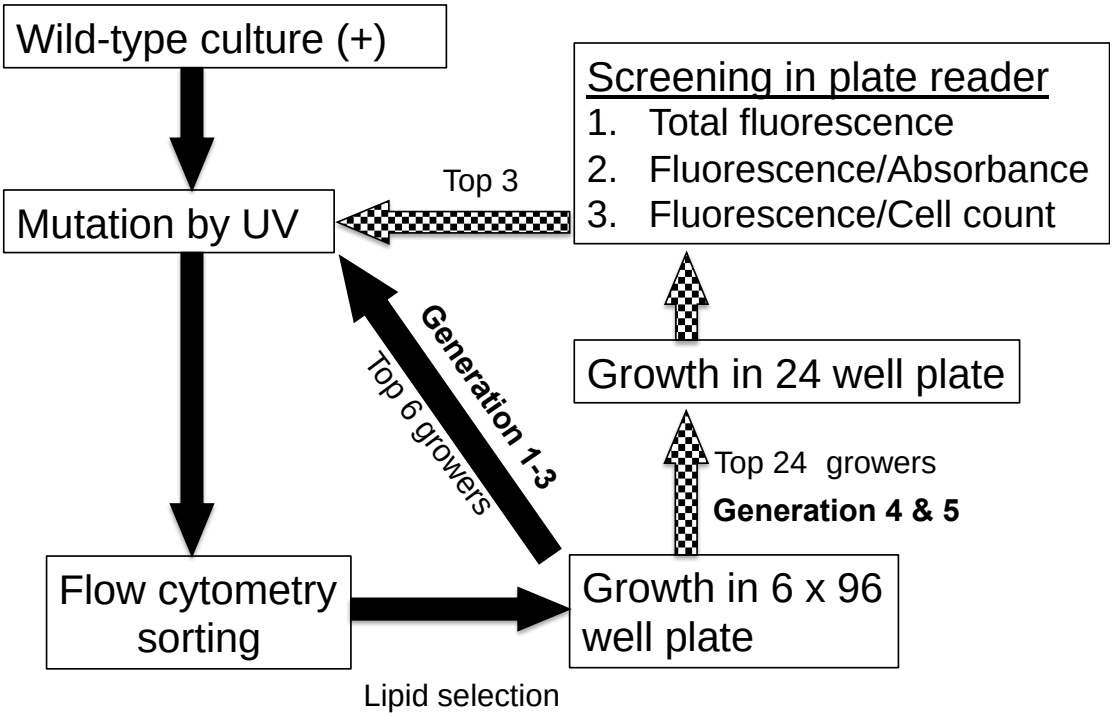

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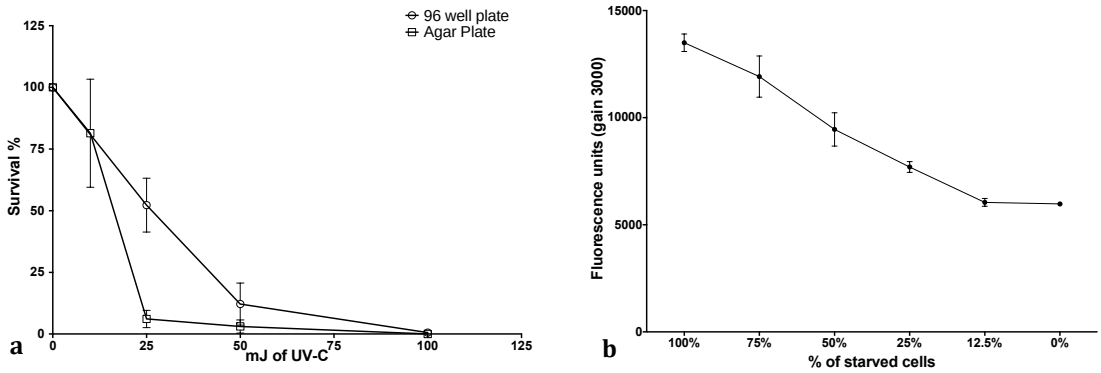

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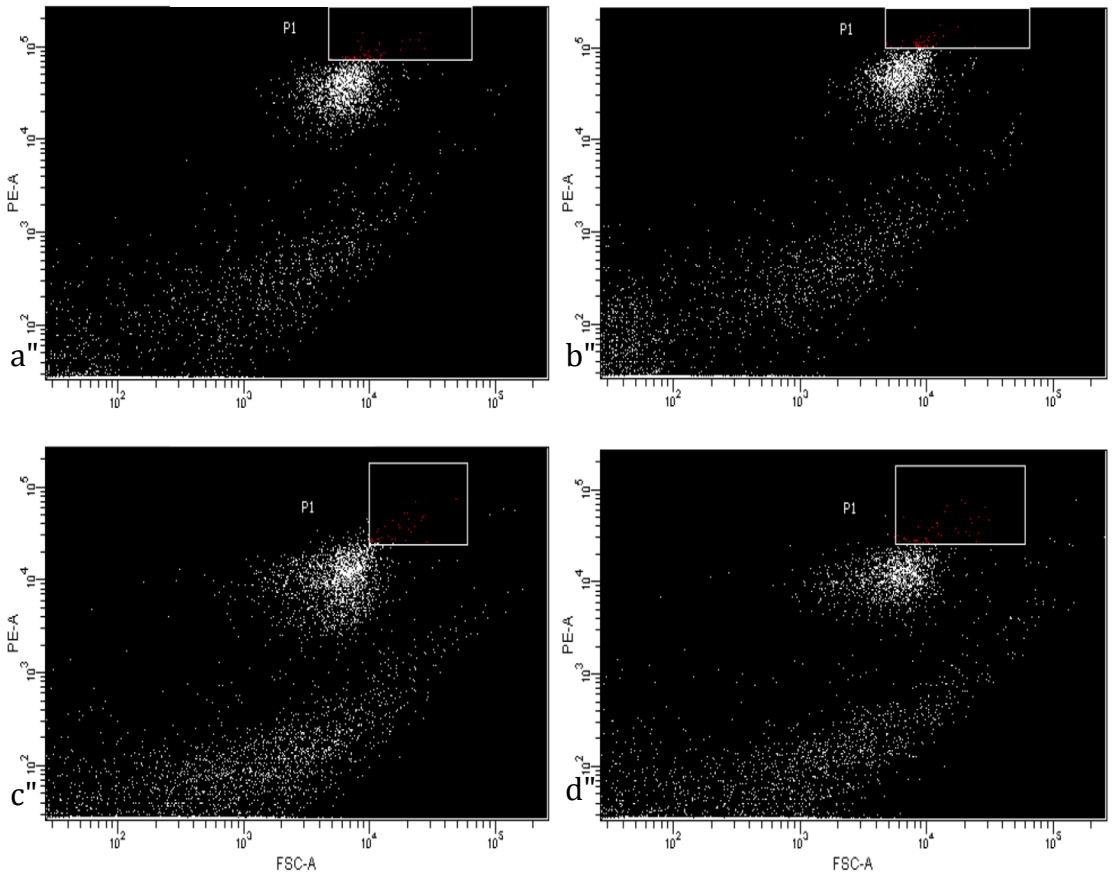

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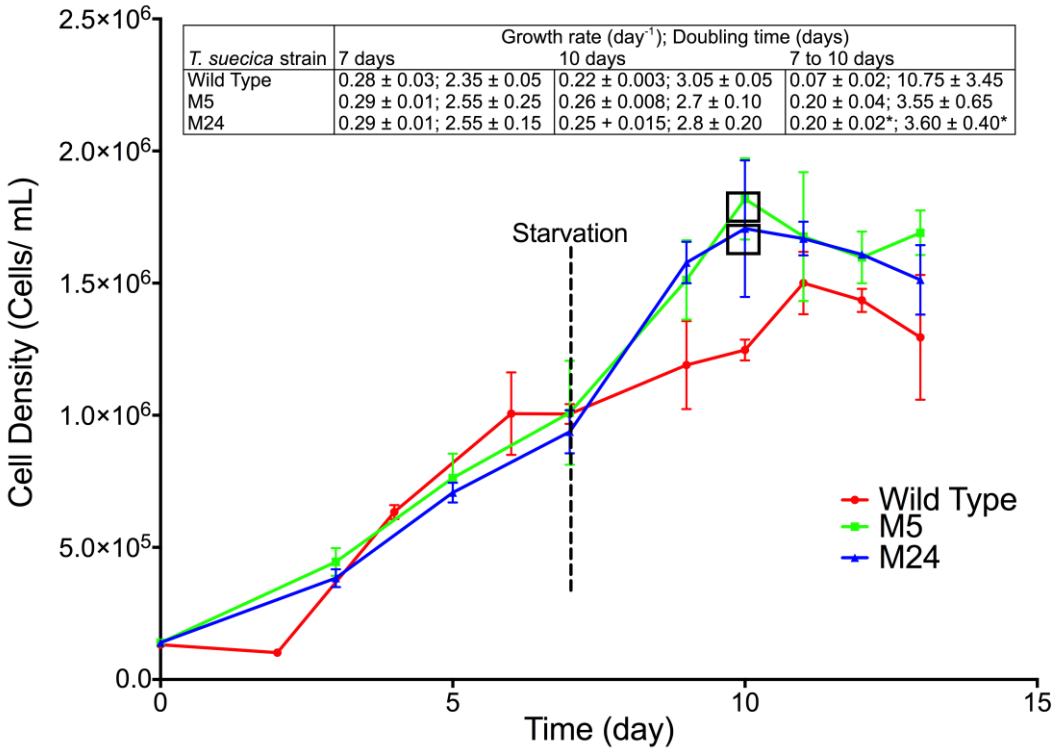

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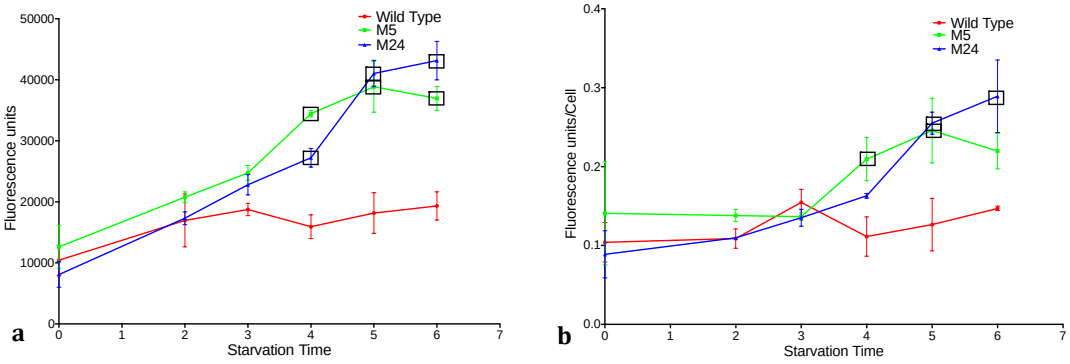

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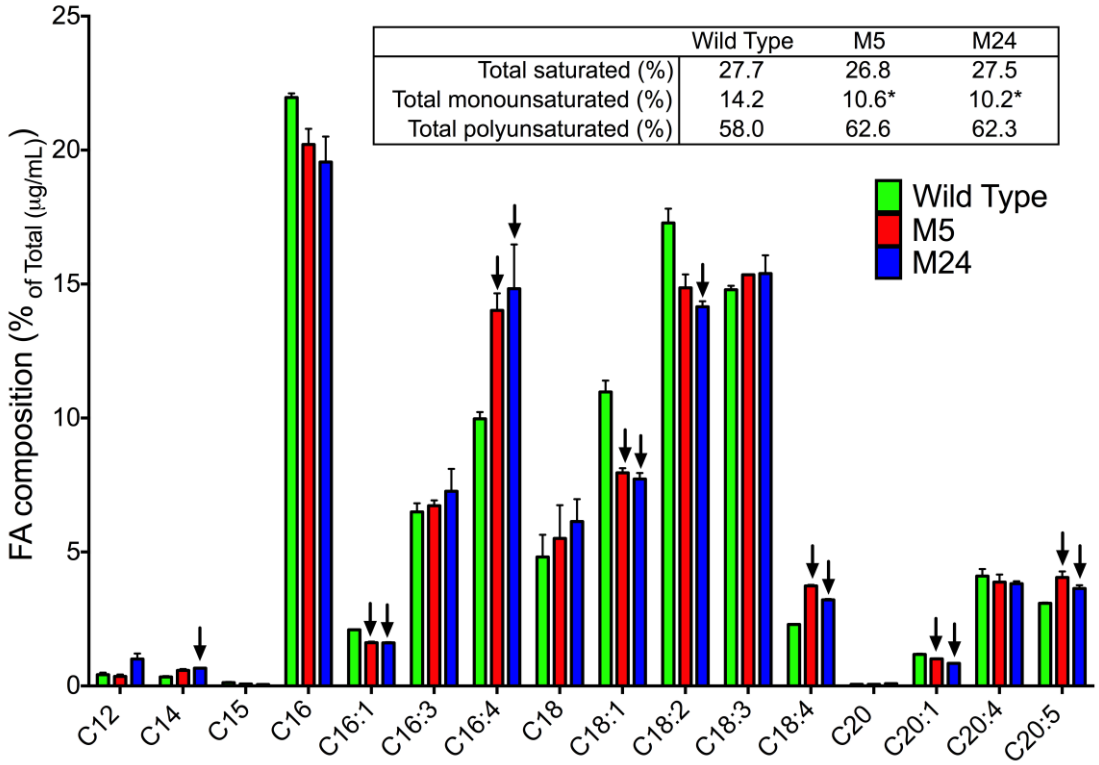

	Wild Type	M5	M24
Total saturated (%)	27.7	26.8	27.5
Total monounsaturated (%)	14.2	10.6*	10.2*
Total polyunsaturated (%)	58.0	62.6	62.3

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